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PRINCIPAL INVESTIGATOR: Doctor Martha R. Stampfer

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Lawrence Berkeley Laboratory
Berkeley, California 94720

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My laboratory has generated a human mammary epithelial cell (HMEC) system for studies on normal and aberrant growth control mechanisms. To facilitate widespread use of HMEC, we have provided cells and cell culture expertise to over 150 laboratories worldwide.

To optimize use of this resource, we developed a complex database for storage and retrieval of information on the HMEC, and newsletters for communication among those using HMEC. The purpose of this grant was to (1) upgrade and expand the features of this database, (2) ensure that all computer and written records were readily understandable to others besides the PI, (3) create written histories of complicated cell lineages and enter them into the database, (4) develop a web site to disseminate information about HMEC culture and to foster communication among laboratories using HMEC.

Most work on the database upgrade was completed in year 1. For years 2 and 3, old records were entered into the database and work was begun compiling cell histories and making written records more accessible. A web site on HMEC was launched in year 2 and has been upgraded in year 4. This work is largely proceeding as planned, although the history compilation will require more time than this granting period.

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FOREWORD

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____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Martha Stanley 9/27/98

PI - Signature Date

Table of Contents

INTRODUCTION

My laboratory's research program has centered on the development and utilization of human mammary epithelial cell (HMEC) cultures. The overall goal of this work has been to generate human epithelial cell systems for studies on the normal mechanisms controlling proliferation and differentiation in human cells, and on how these normal processes may become altered as a result of immortal and malignant transformation. Included in this goal has been the desire to facilitate widespread use of human epithelial cells for molecular and biochemical studies. Therefore, we have endeavored to develop a system that is relatively easy to use, and can provide large quantities of well-characterized, uniform cell populations. We have already provided cells and cell culture expertise to over 150 laboratories worldwide, and new requests continue to be received.

During the past 20 years, we developed an HMEC bank which contains the following types of material: (1) primary cells (frozen as epithelial organoids or cell clumps) from reduction mammoplasties, tumors, non-tumor mastectomy tissue, benign tumors, and gynecomastias from nearly 200 individuals; (2) higher passage pools of single cells from the above tissue types; (3) cells from reduction mammoplasty specimens that have been exposed to benzo(a)pyrene and have acquired extended life in culture; (4) the immortally transformed 184A1 and 184B5 cell lines, including clonal isolates, and spontaneous and carcinogen induced variants of these lines; (5) malignantly transformed derivatives of 184A1 and 184B5. We have also generated three new immortally transformed cell lines (184AA2, 184AA3, 184AA4), and oncogene exposed (HPV16 E6, HPV16 E7, SV40T) derivatives of 184A1.

The widespread usage of these many HMEC types necessitated development of an appropriate database for information storage and retrieval. A 4th Dimension Database was developed in 1987. One purpose of this infrastructure grant was to upgrade and expand the features of this database, and to ensure that all computer and written records can be readily understandable to others besides the PI. The second major purpose of this grant was to set up a web site to disseminate information about my HMEC resources and to facilitate networking among laboratories using HMEC.

BODY

With reference to the specific aims of the proposal:

Update computer database

In Year One, as scheduled, we completed most of the work on the 4th Dimension Database upgrade. These changes have thus far have worked as intended, so that no further *major* changes appear necessary. Minor corrections and changes have been made on an ongoing basis. Further small changes will still need to be made on an ongoing basis. Further work on documentation of use of the Database is still necessary.

Update information in the database

As scheduled for Years One and Two, the old records have been added to the database, and this work is completed.

We have continued checking the actual inventory in the freezers with what exists in computer records. Because this is difficult tedious work, it has moved slowly on an ongoing basis and is not yet completed.

Update written records on cell cultures

This was a major effort for Years Two and Three. The goal here was to review old records to (1) rewrite when necessary to make intelligible; (2) distill histories which can be entered into the computer. In reviewing the amount of material that would benefit from update and clarification, it became obvious that this is going to be a long-term ongoing project, extending well beyond the time frame of this grant. I therefore chose to focus on the most important and worst written records, which were rewritten in a way that others in the lab attested to being comprehensible. These distilled histories were added to the database. We have also been more careful to keep generally intelligible records and write up distilled histories on an ongoing basis. However, it is clear that the goal of completing the rewrite of all our old records was not realistic within the time frame of this grant. I remain aware that this work still needs to

be done, as much of what has been done in my laboratory has not been documented in a form that would be easily understood to other besides myself. Therefore, this work will need to be continued on an ongoing basis under the auspices of my other funding sources.

Another aspect of this work was to go through our 18 notebook files of written records to ensure that records are filed in the appropriate places (many were not). This work has been completed, although constant vigilance is still required to prevent further incorrect filings. No major problems have been encountered.

Set up an Email network and communications network

This has been the main focus of the no-cost extension for Year Four. My initial goal was to post on the Internet most of the information that I previously needed to send to or talk about with other investigators. In years 2-3 we launched our web site (http://www.lbl.gov/~mrgs) with information on cell types available, derivation of HMEC cultures, procedures for HMEC growth, a list of investigators using HMEC, and legal forms which could be downloaded. Since most of this work was done in Year Two, it was now necessary to do a major upgrade on this material. This work has been largely completed, and is currently in the process of being prepared by the work-study student for posting within the next few weeks. We have included as part of the web site an extensive review of HMEC culture and experiments performed in our laboratory using HMEC. We have been gratified by the many positive responses the web site has already received for providing this extensive information. We hope the upgraded version will be a further improvement. As part of the updated version, we have also included some comments on our view of the current state of cell culture, and use of cell cultures for cancer research. We hope this may stimulate thoughtful discussion about some of the issues raised. A printed version of this material is included as Appendix A. The web site still requires an update of the investigators list. This work is in progress and will be completed this year using other funding support.

The development of this web site has also been of great benefit to myself. It has both relieved me of the requirement for many individual mailings and conversations about HMEC culture detail and has allowed other investigators to have a better idea of HMEC culture before discussing their scientific needs with me.

We were also able to make the web site at least partly interactive. A group mailing list was developed such that anyone on the mailing list could send and respond to queries from anyone else on the mailing list. This feature has been used by several of the investigators on the list. However, due to changes in the LBNL network infrastructure, we will need to revise this aspect of the program in the next year, using other funding support.

For the future, there is still much more information I would like to post on the web site about HMEC procedures, and unpublished data from my lab and others that would be generally useful to those working with HMEC. I will also need to update the review of the HMEC system on the web site on an ongoing basis.

General provision of HMEC and information about HMEC

Although this is not a stated task of this grant, I realize that a significant percentage of my time is engaged in assisting other investigators with their usage of HMEC. I believe this is a very cost effective situation for general research in human breast cell biology, and a role I am comfortable doing. I receive around 1-4 requests/queries per week about HMEC via Email, fax, or telephone. In the past year, we sent 44 shipments of cells to 32 different investigators. There are also many additional laboratories using these cells on an ongoing basis. I also respond to many queries on HMEC use, biology, and problems with cell culture, from a variety of investigators, some of whom have obtained HMEC from Clonetics or cell repositories (Coriell, ATCC), or are interested in beginning studies with HMEC. This ongoing interest reminds me of the need to ensure that our cells, and information about them, can be readily accessible. It is difficult to predict what will be needed in the future, however, the present suggests that getting my HMEC records and database in user-friendly shape is a task I should continue.

CONCLUSIONS

The infrastructure work supported by this grant has, for the most part, proceeded as planned. I believe it has been an excellent way, at low cost, to improve the scientific infrastructure for use of HMEC. My database underwent a major upgrade, a web site was launched and is now being upgraded, my record keeping has seen major improvements. The main unfinished task lies in better documenting of the older work in the development of my HMEC cultures, so that it is more intelligible to members of my laboratory. I have realized that reviewing and redoing the cell histories is a much more formidable task than originally anticipated. Additionally, the checking of the freezer inventory has not been completed. Those tasks will still need to be addressed using other funding sources, as will the ongoing updating of the web site and small ongoing changes in the database program. The support of the DOD funding mechanism has been invaluable in allowing the kind of infrastructure work for which it is often difficult to obtain direct funding.

Personnel List

Martha Stampfer, PI
Claudia Madison, computer programmer
Tod Wolfarth, work-study student webmaster
Tony Rose, work-study student webmaster

APPENDIX A: HMEC WEB SITE

WELCOME
HUMAN MAMMARY CELL TYPES AVAILABLE FOR DISTRIBUTION
REVIEW OF HUMAN MAMMARY EPITHELIAL CELL (HMEC) CULTURE SYSTEM PROCEDURES
INVESTIGATOR LIST
LEGAL FORMS
PREVIOUS NEWSLETTERS

WELCOME

This web site provides information on human mammary cell resources that have been developed at my laboratory at the Lawrence Berkeley National Laboratory, University of California. These cells are available for distribution. Information is provided on the cell types available, the derivation of these cells, methods for appropriate use of the cells, and some results which have been obtained in this system. Additionally, a listing is provided of other researchers working on this or related human mammary systems.

As part of my ongoing research program since 1976, my laboratory has developed and stored frozen a variety of normal, benign, tumor-derived, and in vitro transformed cultures of human mammary epithelial cells (HMEC). In the interest of facilitating scientific investigations using HMEC, our laboratory makes these cells available to other researchers. We are not a commercial or official cell bank, and we receive only minor financial support for this service. Please keep in mind the limits of what we can do. Details describing the cell types available can be found in the sections referred to under "**REVIEW OF HMEC CULTURE SYSTEM**".

I prefer talking with individual investigators interested in using HMEC. This gives both of us a better understanding of what is most appropriate for the scientific questions being asked. If you want cells, you will be asked to send a brief (1 page) letter describing your planned experiments, and indicating that (1) you will keep me informed of results or major changes in planned experiments; (2) you will not give the cells to others without my permission. There are also legal forms from the University of California for you and your institution to sign and return. I will **require** a FedEx number or equivalent to charge the costs of shipping the cells. For shipments outside the US, you will need to ensure that all proper customs forms and delivery arrangements are made.

I can be reached as follows:
Martha Stampfer, Lawrence Berkeley National Laboratory, 1 Cyclotron Road, Bldg. 934, Berkeley CA 94720. Phone-office: 510 486-7273; Phone-lab: 510 486-4558; FAX: 510 486-5735; EMail: mrgs@mh1.lbl.gov.

HUMAN MAMMARY CELL TYPES AVAILABLE FOR DISTRIBUTION

Cells are sent frozen (dry ice) in ampoules containing 5 x 10-5 or 10-6 cells. Under **special** circumstances, we can arrange to send growing cells in flasks. (see "**REVIEW section VII.**" for more information on cell shipments)

FINITE LIFESPAN HMEC (section I.)

1. Reduction mammoplasty derived HMEC grown in serum free (MCDB 170 like) medium
Large quantities of post-selection cells are available around passages 7-10 that senesce around passages 15-25 (about 3 population doublings per passage). These cells are routinely available from women of different ages. I most commonly distribute cells from specimens 184, 48R, 239, and 161 (see section I.C. figure 1).

2. Reduction mammoplasty derived HMEC grown in a serum containing (MM like) medium

Limited quantities of cell are available from several individuals. Cells are supplied at 2nd or 3rd passage; most growth ceases around passages 4-5. They contain a greater range of phenotypes than the post-selection MCDB 170 grown cells. I most commonly distribute cells from specimens 184, 48, and 161.

3. Cells from non reduction mammoplasty tissues (i.e., mastectomies; benign tumors, gynecomastias
Limited quantities are available. Cells grown from tumor tissues in MCDB 170 are not reflective of most tumor cells. Talk with me directly about these cells.

FINITE LIFESPAN HUMAN MAMMARY FIBROBLAST CELLS (section I.)

We have available stocks of fibroblast cells from several reduction mammoplasty specimens for which HMEC are available, particularly specimens 184, 48, and 161. These cells are grown in a serum containing medium. In theory, fibroblast stocks can be obtained from any of our specimens, including the mastectomy derived tissues, but we have not grown up stocks to distribute from more than a few. Frozen cells are available around passages 4-6, and they senesce around passages 10-20 (2-3 population doublings per passage).

PRIMARY TISSUES (section I. AB.)

We have very limited quantities of frozen organoids, and are therefore very reluctant to distribute any of this material, but will make exceptions for specific studies. More primary tissue is available from reduction mammoplasties than mastectomies. Talk with me directly about these cells.

IMMORTALLY TRANSFORMED CELL LINES (sections II. & III.)

1. 184A1

This cell line of indefinite lifespan is available for distribution. For some purposes, it may be preferable to have early or later passage cells[]. Clonal isolates are also available. These cells are wild type for p53, and RB, and are not anchorage independent or tumorigenic.

More limited quantities of cells are available which have been selected for loss of specific nutritional requirements.

184A1 retrovirally infected with either the HPV16 -E6, -E7, SV40T, or E1A genes are also available. Talk with me directly about these cells.

2. 184B5

This cell line of indefinite lifespan is available for distribution. For some purposes, it may be preferable to have early or later passage cells. Clonal isolates are also available. These cells are wild type for p53, and RB, and are not anchorage independent or tumorigenic.

More limited quantities of cells are available which have been selected for loss of specific nutritional requirements.

More limited quantities are available of cells which have been transfected with erbB-2 and rendered anchorage independent..

3. 184AA4

This is a newly derived cell line of indefinite lifespan derived from the same extended life precursor population as 184A1. These cells are wild type for p53, and RB, and have not been tested for anchorage independence or tumorigenicity.

4. 184AA2 and 184AA3

These are newly derived cell lines of indefinite lifespan derived from the same extended life precursor population as 184A1. These cells are p53-/- and wild type for RB, and have not been tested for anchorage independence or tumorigenicity.

5. 184-E6

This is specimen 184 post-selection cells retrovirally infected with the HPV16 E6 gene and rendered immortally transformed. Talk with me directly about these cells.

CARCINOGEN EXPOSED EXTENDED LIFE CULTURES (section II.A. and figure 3)

Very limited quantities of benzo(a)pyrene treated specimen 184 extended life cultures, including the extended life precursors of the lines 184A1 (184Aa) and 184B5 (184Be), are available. Talk with me directly about these cells.

REVIEW OF HMEC CULTURE SYSTEM

REVIEW OF HMEC CULTURE SYSTEM

Introduction

The following reviews the origins and characterization of the HMEC system developed in my lab and those of co-workers. This information will be periodically updated. It includes more than you'll probably ever want to know, but hopefully, someone will find each tidbit valuable and consequently not need to query me on that subject. It also includes my personal opinions about HMEC biology and cell culture usage (**III. E. 3.**). I welcome feedback on how this information can be presented most usefully. Some of this information was presented in my four Newsletters from 1987-1989.)

To put this information in context, my long-term goal (since 1976!) has been to develop an HMEC system that could be used to study the **normal** mechanisms controlling proliferation and differentiation in human cells, and to understand how these **normal** processes become altered as a result of immortal and malignant transformation. Guiding this work was the desire to facilitate widespread use of human epithelial cells for molecular and cellular biology studies, i.e., the hope was that HMEC would seem a reasonable alternative to fibroblasts, or tumor cell lines, or non-human cells. Therefore, I tried to develop a system that is relatively easy to use, can provide large quantities of uniform cell populations, and is relatively well-defined. I realize that "relatively easy" can be in the eyes of the beholder, and for some people HMEC will still seem difficult relative to HeLa or 3T3. While HMEC may require a little more effort, really, they are very easy to grow once you get the hang of it. What is needed is careful attention to proper tissue culture procedures, a basic understanding of the cell system, and a "feel for the organisms". Cells are living creatures, with some resemblance to children - they can behave as if they have minds of their own. As Dick Ham has often said, sometimes what is most important is just to "listen to your cells". The rewards are being able to use cells much closer to relevant human processes. Normal finite lifespan HMEC allow you to study growth control in cells with normal human growth control mechanisms.

In developing and promoting the use of the HMEC system, I have been influenced by the following assumptions: (1) Prior knowledge of what constitutes normal cell behavior is necessary to determine what constitutes abnormal and deranged processes, e.g., if you want to say that something you are studying is a property of a transformed cell, you need also to look at the normal cells. (2) Understanding normal and aberrant human epithelial cell growth control and differentiation will ultimately require examination of human epithelial cells. Non-human and non-epithelial cell studies may provide valuable information and suggest areas of research. However, the many differences which are known to exist between these cell types in culture as well as in whole body physiology indicate that only examination of the cells in question will give an accurate description of those cells' behavior. I believe this is especially true in the area of carcinogenesis, where, e.g., major differences in control of telomerase expression between human and rodent cells result in significant differences in the transformation process (see Section III.). (3) In a situation where whole animal experiments are not possible (i.e., with human cells), the next best option is to develop culture systems that can as accurately as possible approximate the in vivo state. I have tried to balance the goal of making the system as amenable as possible to widespread use, with the goal of trying to optimize the system to reflect in vivo biology. The result is considerably less than ideal in terms of in vivo approximation. Normal and aberrant cellular processes in vivo involve complex interactions of polarized cells within three dimensional organ systems. Single cell types growing on plastic are not that! Consequently, it's important to remember the limitations of this culture system. I believe it is valuable that work continue in developing culture systems that more accurately mimic in vivo cell-cell and cell-matrix interactions.

Since fostering widespread usage of HMEC has been one of my long-term goals, I have tried to make cells available to other interested investigators. I have found it helpful to talk to people individually to understand more precisely their scientific needs and goals in using HMEC. Checking through the relevant parts of this web site information first can provide a sense of what is available and known. While distributing cells is part of what I enjoy doing, please keep in mind that this is a non-commercial, non-official, personally-run cell bank, and I and my technician are also doing many other things. I appreciate it when shipment of cells is made as easy as possible for me.

Nomenclature notes:
"**primary**" refers to cells the first time they are placed in culture (e.g., outgrowths from organoids). Cells which have been subcultured are no longer primaries and should not be described as primary culture (this is a common error). I refer to higher passage cultures of normal finite lifespan HMEC as **strains** with **long-term growth potential** in culture. In technical tissue culture parlance, they could be called cell lines once subcultured, but I find this usage confusing and only use "**cell line**" to refer to cells with indefinite growth potential (i.e., immortal). "**extended life**" refers to cells which grow longer than normal as a result of some abnormal in vitro exposure; e.g., chemical carcinogens or oncogenes. "extended life" should not be used to refer to the HMEC strains with long-term growth since this growth is normal.

I. Derivation of HMEC Cultures

I. A. Tissue Procurement

We have obtained our human mammary cells from a variety of sources, mostly surgical discard material. What we refer to as normal HMEC is derived from reduction mammoplasty tissues. Women undergoing reduction mammoplasty operations do not have any known epithelial pathology per se (their breasts contain the same amount of epithelial cells as is present in smaller breasts, but they have much more adipose tissue). Their breast tissues do show the range of pathologies generally found in women of the same age (e.g., it may be described as containing mild to atypical hyperplasia, or fibrocystic disease). There is always the possibility that women with such large fat deposits in their breasts could have some abnormality in some aspect of their metabolism. Because large portions of the breast are removed, with minimal need for pathology evaluation, considerable quantities of cells from the same individual are made available from each reduction mammoplasty.

The other major source of tissues comes from mastectomies. Usually the amount of tumor tissue available for culture is small, due to the need for clinical evaluation of the tumor. Larger amounts of the non-tumor peripheral tissue is available. This can be particularly useful in providing matched pairs of tumor and non-tumor tissue from the same person. However, I do not consider peripheral mastectomy tissue as normal, as there is always the possibility of tumor field effects, microtumors within this tissue, field effects from some environmental conditions predisposing to tumors, and inherent genetic abnormalities. For some of the same reasons, I would not view as normal the tissues we have obtained from contralateral mastectomy - tissues removed from the breast contralateral to a tumor-bearing breast for prophylactic or cosmetic purposes.

Additional surgical tissues are obtained from benign conditions: fibroadenomas (which are not thought to be pre-malignant); fibrocystic tissues (which under some conditions could indicate an increased likelihood of tumor development); gynecomastias, which are benign hyperplasias in male breast tissue.

We also have a few samples of tissues from other conditions. We have two subcutaneous mastectomy tissues. These operations are generally performed because of extensive fibrocystic disease, and in the two samples I processed, the consistency of the tissue appeared grossly abnormal (hard and fibrous) compared to reduction mammoplasties. We have two non-tumor peripheral tissues from breasts that had sarcomas.

Another, non-surgical source of HMEC is from breast fluids. A small number of cells can be obtained from nipple secretions of around 50% of women, and larger volumes are available from lactational fluids. Our original publication in 1980 actually utilized cells from nipple secretions. Cells from milk are valuable as a source of functionally differentiated cells. We have only used these for specific purposes and do not have supplies to distribute.

I. B. Tissue Processing (references: Stampfer et al. 1980; Stampfer 1985 - gives procedure details)

Most of the surgically derived tissues are processed by gross selection of epithelial material followed by digestion for 24-72 hrs at 37°C with collagenase and hyaluronidase. This leaves nearly pure epithelial clumps (termed organoids) which can be separated from the rest of the digested material by collection on filters with pores of fixed size. The organoids can be stored frozen in liquid nitrogen (for at least 21 years - the time since I started this). Material in the filtrate usually contains mainly fibroblastic type cells, and is a good source of matched fibroblasts from the same individual.

The small pieces of tumor tissue are generally not structured in organoids. Digestion for 24 hrs can yield small epithelial clusters and the filtrate may contain many of the single tumor cells. This method is probably not the best available for obtaining tumor cells for culture. It is what was used for the samples that I have stored frozen.

Table 1 gives an idea of what and how many primary tissues we collected and processed.

Table 1: Bank of Primary HMEC Tissue

Tissue Source	# Specimens	Age Range	Median # Ampoules
Reduction Mammoplasty	49	15-66	30

Mastectomy			
carcinoma	57	29-93	5
peripheral non-tumor	43	24-87	8
contralateral	6	42-77	10
Biopsy (benign tumors)	9	13-47	5
Gynecomastia	6	17-57	9

This represents the amount of tissues as originally collected, rather than current inventory levels. We also have the filtrate material for each specimen, from which, in most cases, fibroblast-like cells can be grown. We are reluctant to give out much primary material, since quantities are limited and we are no longer processing these tissues, but small amounts may be available if essential, particularly from the reduction mammoplasties.

I. C. Media for, and growth of HMEC (references: Stampfer et al. 1980; Stampfer, 1982; Hammond et al. 1984; Stampfer 1985)

When I started working with HMEC in 1977, I first developed the MM medium (see **PROCEDURES** for composition of and growth of cells in MM). This medium has a 1:1 DME:F12 base, plus conditioned media from other cell lines, a variety of growth factors, and 0.5% fresh FCS. HMEC obtained from reduction mammoplasties displayed active growth for 2-5 passages. The cultures showed a mixed morphology, with larger, flatter non-dividing cells eventually outnumbering the smaller dividing cells with a cobblestone morphology. I have also employed a number of variations on the MM theme, e.g., with and without a cAMP stimulator (cholera toxin), without the conditioned medium (designated MM4), or without particular growth factors.

While MM provided only a limited amount of cells, this was sufficient to perform many types of experiments. It also provided enough cells to begin more systematic studies on optimizing media for growth of HMEC. This work was done by Susan Hammond in Dick Ham's laboratory, the result of which was the development of the serum-free MCDB 170 medium in 1984. This has a base in which the components have been optimized for HMEC growth, plus a variety of serum-free supplements (see **PROCEDURES** for composition of and growth of cells in MCDB 170). The only undefined element is bovine pituitary extract. When organoids are placed in MCDB 170, there is initial active cell division for 2-3 passages of cobblestone appearing cells. These cells gradually change morphology, becoming larger, flatter, striated, with irregular edges and reduced proliferative capacity. As these larger cells cease growth and die, a small (i.e., a 60 mm dish seeded with 1.5 x 10^5 cells may show 1-10 areas of active growth) number of cells with the cobblestone morphology eventually show proliferative capacity and soon dominate the culture. I have called this process, whereby only a small fraction of the cells grown in MCDB 170 display long-term growth potential, **self-selection**. We now know that the post-selection cells have downregulated expression of the cyclin dependent kinase inhibitor (CKI) p16. Post-selection cells maintain growth for an additional 7-24 passages (approximately 45-100 population doublings in total), depending upon the individual reduction mammoplasty specimen. At senescence, they appear flatter and more vacuolated, while retaining the cobblestone epithelial morphology. Self-selection can also be observed in primary cultures which are subjected to repeated partial trypsinization, a process wherein approximately 50% of the cells are removed and the remaining cells allowed to regrow. After about 10 partial trypsinizations, most of the cells remaining in the dish display the flat, striated, morphology and cease division. However, nearly every organoid patch also gives rise to areas of the growing cobblestone cells, indicating a widespread distribution of the cell type with long-term growth potential. **NOTE**: if you are trying to take cells through the self-selection process, dishes with the large flat cells may sit there for weeks before the smaller cells become obvious. I suspect that this implies that more is happening than the outgrowth of a pre-existing p16 non-expressing population, but we have never investigated this phenomena in depth. **NOTE**: partial trypsinization is a way to obtain more good-growing secondary cultures from primary cultures than if the primaries were fully subcultured. For some reason, the cells in the primaries remain much more vigorous for a longer time period. Perhaps this is due to some mixtures in the primary cell population, or some extracellular matrix material - this question has always intrigued me but it has also never been investigated in depth.

Most of the normal HMEC which I make available (as well as the commercially available HMEC from Clonetics) represent these post-selection cells which display long-term growth in MCDB 170. These cells are particularly useful in molecular and biochemical studies since they provide a virtually unlimited supply of uniform batches of normal human epithelial cells. Thus, experiments can be repeated using cells from both the same frozen batch, as well as from the same individual. These post-selection cells grow rapidly (doubling times of 18-24 hrs) and will grow clonally with 15-50% colony forming efficiency. However, it is important to remember that the cells with long-term growth potential represent a selected subpopulation of the mammary epithelial cells placed in culture (see below). We have grown a limited number of our frozen primary organoids specimens in MCDB 170, generating large pools of frozen cells for use in our laboratory, as well as for distribution to others. We have thus far grown up cells from 12 reduction mammoplasty tissues, 8 mastectomy tissues (6 tumor tissues, 5 non-tumor, 1 contralateral), and 1 gynecomastia. Figure 1 illustrates the long-term growth potential of cells from each of these individuals. **NOTE**: we have not observed a single instance of spontaneous escape from senescence in the HMEC grown under these conditions. In general, cells from the same individual senesced around the same passage, but there were some exceptions. The following explains how and why we kept track of this information.

Since the post-selection cells in our large freeze-downs may be derived from a small number of p16 non-expressing cells, it was possible that a few cell outgrowths with some unusual quality could influence a given freeze-down pool. As a consequence, we gradually (and informally) developed a nomenclature to keep track of the origins of a given cell pool. At the first level, we started using symbols to indicate every time we started a new primary organoid ampoule from the same individual. These were easy-to-write symbols with which to label the dish (e.g., heart ♥, infinity ∞, birdie "V", spiral "@", etc.). These are now officially registered in our computer records as **FreezeDownSymbol (FDS)**. Subsequently, we realized that it might be important to also keep track of cell populations coming from different pools of post-selection cells. Each selection pool can be thought of as a different substrate "batch", with the possibility that there might be batch differences. So our FDS may be followed by an indication of **"selection" batch** (e.g., ♥Δ, ∞3, @K, @L, etc.). These are the symbols present in Figure 1. Visually, cells from the same individual, regardless of batch, tend to have the same characteristic appearance, while we do notice interindividual morphologic differences (see Figure 2). **NOTE**: the most common batch of cells from specimen 184 that I distribute is @K, which senesces around passage 22, whereas most other batches from specimen 184 senesce around passage 18.

Figure 1. Growth capacity of HMEC in MCDB170 medium. I stopped adding information to this graph several years ago, but this gives the general idea and includes the FDS and selection batch of the cells I most frequently distribute. You can use this to see the expected passage where the cells senesce. Primary cultures obtained from reduction mammoplasties (top two rows) and mastectomies (T= tumor tissue, P= non-tumor tissue from tumor-bearing breast, C= contralateral) were initiated and subcultured with about 8-10 fold amplification per passage. Bottom horizontal lines indicate passage level of initiation of frozen ampoules. Top horizontal lines indicate passage level of no net increase in cell numbers (i.e., replicative senescence is viable; some growth does continue as long as cultures are maintained). Internal horizontal lines indicate that cultures were frozen and reinitiated at that passage. Same shading indicate cells derived from the same "selection". Asterisks indicates cells exposed to a cAMP stimulator during selection. For specimen 184 "V", cultures were initiated from the same primary ampoule but taken through selection with three different cAMP stimulators (cholera toxin, isoproterenol, prostaglandin E_1). In a few cases, (indicated by a different shading in primary culture), the tumor cultures were grown in MM in primary culture.

[PS: the names of the symbols shown, in order of appearance, are: 161- heart, triangle, newmoon, yinyang, infinity; 184- birdie, spiral (not shown are aleph, cross, lollipop, ecology; flower); 48- silver, orange, pink (not shown are blue, tulip); 172- icecream, lollipop, diamond; 195L- teardrop, pumpkin; 186T- heartbrk, sunrise]

Other information which may be gleaned from these data: (1) There does not appear to be any loss in viability due to multiple freeze-thaws; (2) There does not appear to be any correlation between growth potential in culture and age of specimen donor. It is possible that some of the differences seen in growth potential could reflect interindividual differences in optimal growth requirements relative to the nutritional formulation of MCDB 170.

Figure 2. Morphology of reduction mammoplasty derived HMEC grown in MCDB 170. Giemsa stained cultures from
(A) 184 p7; (top image)
(B) 172 p13; (middle image)
(C) 161 p9; (bottom image)

I. D. Characterization of Tissue-derived HMEC (references: Taylor-Papadimitriou et al., 1989, Stampfer & Yaswen, 1992)

Since a main goal of studying cells in vitro is to gain understanding of in vivo processes, we have considered it extremely important to characterize the HMEC grown in culture with reference to what is known about human mammary cells in the body. Unlike other organ systems, HMEC in culture (with the exception of lactational fluids and very rare surgical specimens) are not obtained from functionally differentiated tissues (i.e., pregnant, lactating, involuting). Consequently, we have not put much effort into examining these cells for features of functional differentiation. The cells we distribute, growing under standard culture conditions, do not express α-lactalbumin or β-casein. We have instead focused on the type of differentiation we termed "maturation", referring to the developmental history of a cell from a proliferative stem cell population to a cell with diminished reproductive capacity to a "terminally differentiated" cell no longer capable of division. We have been particularly interested in this pathway because human breast tumor cells in vivo and tumor derived cell lines almost uniformly express the phenotype of the most mature normal HMEC in vivo.

The mammary gland consists of pseudostratified epithelia, with a basal layer resting upon a basement membrane and an apical layer facing the lumen of the ducts and alveoli. The basal layer of cells does not contact the lumen, whereas the apical layer may contact the basement membrane as well as the lumen. Apical cells display a polarized morphology, with microvilli at the luminal side. The myoepithelial cells, which contain muscle-like myofilaments, and which contract upon appropriate hormonal stimuli to cause expulsion of milk, lie in the basal layer of cells. Based upon examination of keratin expression and other marker antigens, it has been proposed for the rodent mammary gland that a stem cell population capable of differentiating into both myoepithelial cells and the apical glandular epithelial cells, also resides in the basal cell layer. The actual maturation lineage of human mammary epithelial cells in vivo has not been clearly defined. Based on the rodent mammary gland, and other epithelial tissues, it is reasonable to hypothesize that the most proliferative epithelial population in vivo lies in the basal layer, or intermediate between basal and luminal layers. Conversely, the luminal cells presumably have reduced proliferative capacity, with the most mature and least proliferative cells being those shed into the lumen (and recovered in nipple aspirations and milk fluids). **NOTE**: I do not equate basal cells with myoepithelial cells, which are specialized differentiated contractile cells. Myoepithelial cells may all be basal, but not all basal cells are myoepithelial. Unless one can demonstrate the presence of myofilaments, I do not think a cell should be referred to as myoepithelial.

A variety of studies from Joyce Taylor-Papamidritriou's group and others (see references at end) have defined properties which can be used to distinguish basal vs. luminal human breast cells, cells during lactation, and tumor cells. In general, mammary basal cells, similar to basal cells in stratified tissues such as the skin, express keratins 5 and 14. α-actin is present and the calmodulin-like protein (CLP) is preferentially found in the basal cell layer. A subpopulation expresses the common mesenchymal intermediate filament, vimentin. Luminal cells express the keratins 8 and 18 found in simple epithelia like the lung; keratin 19 shows variable expression and the 19 positive cells probably represent the most mature population. In culture, keratin 19 expressing cells display very little proliferative potential. Expression of specific epitopes of a polymorphic epithelial mucin (PEM) is localized to luminal cells in vivo. Cells in the resting gland are weakly PEM positive, whereas cells from lactating glands may express higher levels of specific mucin epitopes. Like keratin 19, high expression of specific PEM epitopes has been correlated with a low proliferative potential in milk derived HMEC in vitro. Only a small fraction (~3-10%) of normal HMEC in vivo show detectable estrogen receptor, and this positive population is preferentially localized in the non-basal layer. It is not clear that the mammary gland contains cells which are terminally differentiated, such as those in the most mature layers of stratified epithelium, since even keratin 19, PEM positive cells have a limited capacity for cell division in vitro.

Unlike what one might intuitively expect, and unlike stratified epithelial tissues, breast tumor cells in vivo and tumor cell lines in vitro almost all have the phenotype of the most mature luminal cell - positive for keratins 19, 8/18, high expression of several PEM epitopes, including those found in the differentiated lactating cells, negative for keratins 5/14, and CLP. The consistency of tumor expression of keratin 19 has been utilized to locate micrometastases in lymph nodes. As normal HMEC with this phenotype show little or no growth in culture, I think this tumor cell phenotype is indicative of some aspect of derangement in growth control. Most tumors also initially have high expression of estrogen receptor, and most are initially negative for vimentin expression, although vimentin is seen in a subset of estrogen receptor negative breast tumor cell lines and tissues. I don't know of a definitive explanation for this tumor cell phenotype, nor has the maturation state of the tumor cell precursor in vivo been clearly established. Benign proliferative tumors and some in situ carcinomas contain keratin 19 negative cells, so a keratin 8/18 positive, 19 negative cell could be the precursor of invasive breast tumors. If so, expression of the keratin 19 and high PEM phenotype in invasive tumors may be a consequence of malignant transformation.

In collaboration with others, we have examined the HMEC grown under our culture conditions for expression of the above phenotypic markers. Primary cultures of normal HMEC grown in MCDB 170 and early passage cultures grown in MM are heterogeneous. Some cells have the basal phenotype: keratin 5/14 positive, PEM negative, and vimentin, CLP and α-actin positive; other cells show the luminal phenotype: keratin 5/14 negative, keratin 8/18/19 positive, PEM positive; and some are in-between (e.g., keratins 5/14/8/18 positive). The cells which initially proliferate in MCDB 170 medium have the basal phenotype. However, post-selection cells begin to express some luminal markers, i. e., keratins 8 and 18 and some PEM epitopes. Expression of these luminal properties increases with continued passage in culture, such that the senescent cells uniformly express these markers. At the same time, expression of the basal keratins 5/14, CLP, and vimentin is not lost. We have not detected keratin 19 or estrogen receptor in the post-selection population. All HMEC examined derived from normal tissues have shown a normal karyotype.

The above results led us to propose that the cells which display long term growth in the serum-free MCDB 170 represent a multipotent stem cell population initially present in the basal layer of the gland. With increasing time in culture, these cells show a partial differentiation towards the luminal phenotype. However, it is a possibility that culture conditions have induced some artifactual phenotypic expression. In particular, growth of cells on impermeable plastic substrates prevents the normal cell-extracellular matrix contacts and precludes the normal development of cellular polarity. Although we now know that the post-selection cells have p16 promoter methylation and no p16 expression, we still do not know if this represents a normal cellular process or an in vitro artifact. **NOTE**: The post-selection cells in MCDB 170 represent a limited phenotype. While they represent a wonderful normal human epithelial finite lifespan cell with which to study questions of growth control, for some experimental purposes, these may not be the best cell types to use. Cells grown in MM type media show the range of in vivo phenotypes. However, they have a much more limited lifespan and we have less of them to distribute. Additionally, although they contain keratin 19 positive cells, these are not the actively dividing cells in the population.

II. In Vitro Transformation of HMEC

II. A. Derivation of Cell Lines 184A1 and 184B5, and Extended Life Cultures (references: Stampfer & Bartley, 1985; Stampfer & Bartley, 1988; Walen & Stampfer, 1989)

One of my main goals in developing a culture system for normal HMEC was to use the normal cells as a basis for in vitro transformation, so that different stages of malignant progression could be compared using cells from one individual. I was interested in using a chemical carcinogen as the agent for transformation because: (1) I wanted to induce random errors; (2) there was a lot of data indicating polycyclic aromatic hydrocarbons (PAH) were good inducers of mammary cancer in rodents, and I wondered if the same could be true for humans; (3) chemicals seemed easier to use than radiation; (4) we performed a series of experiments indicating that HMEC were very efficient at converting PAH procarcinogens to their active form (see section VI. A.).

Three sets of experiments were performed in the early 1980's using primary cultures from normal HMEC specimen 184 organoids. The three separate original cultures had the FreezeDownSymbols: "aleph"(A),

"cross"(C), and "birdie"(B). In each case, cells in at least 2 T-25s were treated for 2 or 3 24 hr periods with 1-2 µg/ml of benzo(a)pyrene (BaP), (a concentration that gave 80% killing) and 2 T-25s were treated as controls. The cells were grown in MM medium, in which 184 normally stops growing by 5th passage. We followed the fate of the treated and control cells both in primary culture (how long growth was maintained in the primary T-25 flasks) and after subculture (the primary flasks were partially trypsinized many times, and after some trypsinizations the removed cells plated and passaged until growth ceased).

Figure 3 shows the fate of these cells and indicates the nomenclature we used to identify Extended Life (EL) cultures and the immortally transformed cell lines. We gave a number to each of the subcultures we passaged in experiments C (cross) and B (birdie); i.e., the B1, B2, B3 etc. that you see in the figure. The EL cultures (i.e., treated cells that kept growing after the controls had stopped growing) derived from each of these subcultures were given the alphabetical equivalent to these numbers, e.g., B5 = Be, C2 = Cb. The immortally transformed lines that developed were given the subculture name, i.e., B5 and A1. We have extremely limited frozen stocks for some of these EL cultures. Subsequently, we grew some of these MM derived EL cultures in MCDB 170, which we found permitted growth for an additional 2-5 passages. Still, we have very limited stocks of EL cells so we're hesitant to distribute them. We would consider specific cases of collaboration or mutual interest, so check with me. The EL cells were notable for their heterogeneity with respect to morphology and growth potential (see Figure 4). Growth often followed a punctuated pattern, with outgrowth (lasting 1-5 passages) of individual patches or colonies within non-growing populations. **NOTE**: we now know that all of these EL cultures tested do not express p16. In only one case, 184Aa, is this due to a detectable mutation. We surmise that in MCDB 170, some cells can spontaneously downregulate p16, while in MM, some cells can downregulation p16 after carcinogen exposure, but rarely if at all spontaneously. Thus, although these carcinogen treated cells are called EL - this is in relation to MM grown controls. When grown in MCDB 170, EL and post-selection cells cease growth at approximately the same telomere length. **NOTE**: most, though not all, of the EL cells had morphologies/growth patterns clearly distinct from anything in the untreated populations. I suspect this implies something about changes which have occurred, which could affect cell-cell or cell-matrix interactions.

Figure 3. Growth of BaP treated specimen 184 in MM. It was conveniently fortuitous that the FDSs chosen for these three experiments started with the first three letters of the alphabet. When published, I presented them in the order A, B, C so it would look intentionally linear, but the actual order of the experiments was A, C, B and their real names are aleph, cross, birdie. The figure follows the fate of the treated (T) and control (C) cells in primary culture and upon subculture. Since several dishes were plated at each subculture, and if growing, their lineages followed independently, more than one kind of growth pattern could be observed at a given passage level. In experiment 184C, cholera toxin was inadvertently omitted from the medium until 22 days after seeding (p5 of subculture C1, p4 of C2, p3 of C3).

Figure 4. Morphology of EL cultures, Giemsa stained.
(A) 184C p8 with mixed growing and non-growing cells throughout the dish;
(B) 184C p6 containing two focal growing areas, one with uniform growth (shown) and one with mixed growing and non-growing cells (not-shown);
(C) 184B p6 with non-growing and actively growing cells in a "hyperplasia" morphologic pattern;
(D) 184C p7 with swirly thumbprint morphology. 184Aa had almost exactly the same appearance when it first showed up as a single patch in 184A p5;

Eventually, almost every EL cell ceased growth. The two exceptions were the appearance of the 184A1 line from the 9th passage 184Aa EL population, and 184B5 from the 6th passage 184Be. 184A1 stood out as a more refractile appearing cell growing more vigorously as "eye-shaped" singlets, compared to the patchier, flatter, less vigorous 184Aa (which died by passage 11). Some cells were transferred to MCDB 170 medium at passage 11 and carried continuously in that medium to passage 105. Cells were also maintained in MM up to passage 69. 184B5 was a sickly looking small tight patch, somewhat more refractile than 184Be, very slow growing, that strongly caught my attention for undefinable reasons. It was first transferred to MCDB 170 at passage 9 and grown to passage 101. Cells maintained in MM were grown to passage 30. It is curious and perhaps indicative of some underlying structure that the first time I saw both these cells, I was sure they were transformed, and I did not have that sense with any other cells in the EL cultures.

Both of these lines show specific clonal karyotypic aberrations, indicating their independent origins from a single cell. Some of the karyotypic abnormalities found in 184B5, e.g., 1q22 breaks and tetrasomy for 1q, are also frequently observed in cells obtained from breast tumors. Upon continued passage in culture, these two lines show some genetic drift (more so in 184B5 than 184A1), but it is relatively minimal compared to that observed in most human breast tumor cell lines. Even at passage 41, 184B5 has clearly identifiable chromosomes and a near pseudodiploid karyotype. Thus, the vast majority of the cell population would be expected to remain karyotypically stable when studied over the course of a few passages in culture, yet the presence of some genetic drift could give rise to rare variants in the cell population. Although 184A1 and 184B5 have an indefinite lifespan, they do not have properties associated with malignant transformation. They do not form tumors in nude mice and they do not express a sustained capacity for anchorage independent growth (AIG), although 184B5 can show a low level of random colony formation. 184B5 has a distinctive morphology, growing in tightly packed patches. An advantage of this is that the fate of single cells can easily be followed without needing to seed at clonal densities (the progeny of a single cell stay attached and make a colony). Early passage conditionally immortal 184A1 cells (see Section III) grew with minimal cell-cell contact at low densities, and showed some morphologic heterogeneity, with the presence of large vacuolated cells. At higher passages, when converted to full immortality, the growth pattern shows more cell-cell association and patchy growth, with few vacuolated cells visible (see Figure 5 and discussion the of conversion process in section III). Since 184A1 and 184B5 are cell lines of indefinite lifespan, I have unlimited supplies to distribute.

Figure 5. Morphology of 184A1 and 184B5, Giemsa stained. All pictures shown at the same magnification.
(A) 184 p9 in MCDB 170;
(B) 184A1 p15 in MCDB 170; note the large vacuolated cells;
(C) 184A1 p42 in MCDB 170;
(D) 184B5 p11 in MCDB 170.

CAUTIONARY NOTE: We were remiss in our earliest tissue culture years in not routinely checking all cells for PPLO contamination. We first started routinely testing for PPLO in 1982, after experiments "A", "C" and "B" were initiated, and the cell lines 184A1 and 184B5 were being maintained in MCDB 170. These lines, as well as other normal and benzo(a)pyrene treated extended life cells growing in MCDB 170, were tested for PPLO by Hoechst stain and growth in agar broth. The results were all negative. About a year later, we took out some of our frozen extended life benzo(a)pyrene treated cells, and placed them in MM. Now our routine Hoechst stain test showed some of them to have foreign DNA, although broth growth was still negative. Transfer of the samples to MCDB 170 generally led to loss of the Hoechst stain positive material within 2 passages. This negative phenotype was retained after transfer back to MM. Two (visually equivalent Hoechst stain positive) samples were sent to Microbiological Associates for assay by agar growth, Hoechst, and strain-specific antibodies. They reported one to be completely negative and the other positive for M.hyorhinis (which doesn't grow in the usual agar broth assay). From our Hoechst stain results, it appears that 184Aa, the EL precursor of 184A1, was positive, while some, but not all of 184Be, the EL precursor of 184B5, may have been positive. We can not say whether this may, or may not, have affected any results. By the Hoechst and Mycotect assay, our current normal HMEC, EL, 184A1, and 184B5 are negative. It was a mistake for us not to have been testing our cells. A take-home message is to be careful to check your cells on a regular basis.

II. B. Derivation of Variants of 184A1 and 184B5 (references Clark et al., 1988; Stampfer & Yaswen, 1992)

One of my original goals for in vitro transformation of HMEC was to obtain malignant transformants from normal HMEC. To be honest, somewhere along the line I realized that although this was important science, I myself was less than enthusiastic about creating malignant cells from normal, and the immortally transformed cells were sufficient for my scientific curiosity (or as I commented, as I got older, the question of immortality seemed more interesting than malignancy). Nonetheless, I did try (and not succeed) to obtain malignancy by exposing the 184A1 and 184B5 lines to further chemical carcinogens, in this case, the direct acting carcinogen, N-nitroso-ethyl-urea (ENU). Others exposed the lines to specific oncogenes, which could lead to cells that were AIG and/or made tumors in nude mice. As part of this work, I analyzed the cells for their nutritional requirements and isolated/developed cell variants with

altered nutritional requirements. The history of these studies and the names/origin of these variants is described below.

II. B. 1. Nutritional Variants

Nomenclature Note: Where spontaneously occurring subpopulations were isolated based on the nutritional composition of the medium, they are designated as **A1N...** and **B5N....** In the case of MM grown subpopulations, the N is followed by a (arbitrary) number; in the case of MCDB170 grown cells, the N is followed by letters indicating what factors were no longer required. Nutritional variants obtained following exposure to ENU are designated **A1ZN...** and **B5ZN...** followed by letters indicating the non-required factors. While I sometimes refer to these nutritional variants by their complete names (e.g.., 184A1N4), for brevity and simplicity sake, it's OK to officially refer to them without the 184 prefix.

The first nutritional variants were isolated (in a non too systematic fashion) from 184A1 growing in MM medium. For general purposes, the only one of these to note is **184A1N4**. 184A1 was seeded at p16 in MM minus the conditioned media and without cholera toxin. Attachment appeared poor but the few patches that were present grew fairly well. After 3 passages, there appeared to be uniform good growth and attachment. These cells were first transferred to MCDB 170 at p28. The karyology of A1N4 indicated that, unlike the pseudodiploid 184A1, A1N4 were aneuploid (near triploid) with only one additional chromosomal marker beyond the 4 seen in the parental 184A1 cells. It is therefore likely that, although not cloned, they represent a clonal population. The A1N4 were used by Robin Clark for malignant transformation with oncogenes.

More systematic isolation of nutritional variants was done in MCDB 170 medium. The existing literature indicates that many transformed cells show reduced nutritional requirements. As part of our initial characterizations of the immortally transformed cell lines, we first compared the requirements of 184, 184A1, and 184B5 for the individual growth factors present in MCDB 170 for short term growth, for long-term culture, and in clonal vs. mass culture (Table 2 and list below). 184A1 and 184B5 showed a few differences from each other and normal HMEC. Both were more dependent upon EGF for growth in mass culture (although EGF independent variants could be isolated) whereas the normal cells could continue to proliferate without EGF (see section III.). All of these HMEC showed a stringent requirement for EGF in clonal culture. 184A1 showed little effect upon removal of hydrocortisone (HC); 184B5 and 184 had greater short-term requirements. All the HMEC had a requirement for BPE for short-term growth. In the long-term experiments, removal of HC or BPE from mass cultures of normal HMEC led to cessation of growth over the course of 1 to 3 passages. Removal of insulin (I) did not prevent continued proliferation, but led to slower growth, a less healthy appearing culture, and earlier senescence. Removal of I from 184A1 and 184B5 also did not prevent continued growth. For more details of the long-term experiments with the cell lines, see the list below. These nutritional variants are available for distribution. My general conclusion from these studies is that 184A1 and 184B5 retain basically normal growth factor dependence (except for 184A1 and HC).

Table 2. Growth Factor Requirements of Normal and Transformed HMEC in MCDB170

	Percentage of Control Cell Growth					
	184		184A1		184B5	
Medium	MC[a]	CFE	MC	CFE	MC	CFE
Complete MCDB 170+IP	100	100	100	100	100	100
minus I	49	47	11	18	26	73
minus HC	36	32	84	88	18	61
minus EGF	86	2	20	0	12	0
minus BPE	15	21	21	24	16	75

[a] Abbreviations used: I, insulin; HC, hydrocortisone; EGF, epidermal growth factor; BPE, bovine pituitary extract; IP, isoproterenol; MC, mass culture growth; CFE, colony forming efficiency. Cells from specimen 184 (p11), and cell lines 184A1 and 184B5 (passages 17-20) were grown in complete MCDB 170 with isoproterenol. For mass culture, cells were subcultured into duplicate 35 mm dishes (5 x 10^4 per dish) in the indicated media. When control cultures were subconfluent or just confluent, all

the cultures were trypsinized and the cells counted by hemocytometer. For clonal cultures, single cells (100-1000) were seeded into triplicate 100 mm dishes. After 10-14 days, cells were stained with Giemsa and colonies greater than 30 cells counted.

List of Spontaneous Nutritional Variants:

184A1:
CAUTION: the nutritional requirement studies were done with 184A1 at passages ≤ 20 and the selection for variants was done with 184A1 around passages 27-32. We now know that these are non-homogeneous conditionally immortal populations (see section III) The results might be different if later passage fully immortal cells were used.

184A1NE: no EGF. For the first 2 passages, growth was slow and selective (a small # of patches). The growth rate was the same as control (+EGF) after 4 passages.
184A1NH: no HC. Slow patchy growth for first passage; growth normal after 2 passages.
184A1NI: no I. Growth was initially slowed, but less selective than -EGF; the cells looked good. Growth rates were normal within 2-4 passages.
184A1NB: no BPE. Little initial growth. Eventually a few patches grew out. After one additional passage the resultant cells grew normally.

184B5:

184B5NE: no EGF. Media first changed at p48. Growth initially slower and selective. It took 7 passages to select a population that looked good and had a normal growth rate. Repeated with cells at p36, after 3 passages of slow, selective growth a good growing population arose.
184B5NH: no HC. Media changed at p34. Growth initially slower but normal after 2 passages.
184B5NI: no I. Media changed at p35. Growth initially slowed but not as selective as -EGF. Growth rates were normal within 4-6 passages.
184B5NB: no BPE. Growth initially slow and selective but not as extreme as 184A1 -BPE. Cells didn't look good and grow normally until after 6 passages.
184B5NIB: no I or BPE. 184B5NB cells were switched at p42 to media without insulin. They grew initially slowly and poorly. Good patches were obvious after 2 passages and growth was normal after 3 passages.

We next examined the effect of removal of multiple growth factors to determine conditions where untreated 184A1 and 184B5 did not yield spontaneous nutritional variants. These were defined as removal of I and EGF, I and BPE, or EGF and BPE for 184A1, and removal of I and EGF, or I and BPE for 184B5. Populations of 184A1 and 184B5 were then tested for their ability to grow in these restrictive media after exposure to ENU concentrations that yielded 80% inhibition of colony forming efficiency (1500 μg/ml for 184A1 and 750 μg/ml for 184B5). Two T-75 flasks each of treated and control cells were exposed to ENU or solvent alone for 2 or 3 consecutive passages. Under a few conditions the ENU treated cells were capable of sustained growth whereas the untreated cell lines quickly ceased growth. The resulting growth factor independent variants may represent a further step in malignant progression. However, they did not show AIG or form tumors in nude mice.

List of ENU-induced Nutritional Variants:

184A1ZNEB: selected in MCDB170 -EGF-BPE. The treated cells had a fair amount of growth (compared to almost nothing in the controls), but most of this faded away after several passages. In one experiment, cells with patchy vigorous growth and a distinctive morphology quickly took over the population, and maintained active growth in this medium. Although not examined, these are presumably clonal.

184B5ZNEI: selected in MCDB170 -EGF-I. The treated cells showed initial widespread, morphologically heterogeneous, growth (compared to very little in the controls). Most of this growth faded after about 5 subcultures but in several cultures growth was maintained. The morphologies are not particularly distinctive and we don't know if these represent clonal cultures.

These variants are available for distribution. For more information on the non-EGF requiring variants, see Figure 7.

II. B. 2. Oncogene Exposed Derivatives (early studies) (references Clark et al., 1988; Stampfer & Yaswen, 1992; Frittitta et al., 1995)

AIG and malignant derivatives of 184A1 and 184B5 were initially obtained with the use of oncogene containing retroviral vectors and viruses, and transfection. In the case of 184A1, A1N4, a clonal derivative with reduced nutritional requirements, was exposed by Robin Clark to the genes for SV40 large T antigen, v-H-ras, and v-mos, singly and in combination. The combination of H-ras and SV40-T led to cells (designated **A1N4-TH**) which formed progressively growing tumors in nude mice and showed AIG. Exposure to v-H-ras (**A1N4-H**) or v-mos (**A1N4-M**) alone led to cells that produced tumors with reduced frequency and longer latency. SV40-T alone (**A1N4-T**) did not yield tumorigenic cells, but did affect the growth factor requirements for anchorage dependent and independent growth. In all cases of oncogene exposure, the resultant cells were capable of proliferation in media that did not support the growth of the parental A1N4 cells. A1N4-TH has a near tetraploid karyotype, which is missing the A1N4 chromosomal marker and contains only one additional clonal chromosomal aberration relative to 184A1. Thus even the malignantly transformed cell line, containing v-H-ras, does not show a very unstable karyotype in terms of gross chromosomal aberrations.

The 184B5 cell line was exposed to v-K-ras (designated **184B5-K**) by Paul Arnstein, yielding cells which were tumorigenic in nude mice, with short latency. However, these tumors did not grow beyond approximately 5 cm diameter. Most of our studies have utilized the culture designated **184B5-KTu**, which was derived from a B5-K tumor resected from a nude mouse and replaced in culture. B5-K and B5KTu do not display AIG.

Although we have some stocks of these cells available, we are not eager to be growing or distributing these cells, as the infection was with non-defective retroviruses. If you really want oncogene exposed 184A1 or 184B5, I suggest you transfect the cells with the oncogenes of your choice. We do now have available 184A1 infected, using defective retroviral vectors, with HPV16 -E6, -E7, or SV40T (see Section III. B.) Alternatively, other investigators may have already infected the cells with your oncogene of choice, and you may query for this information (see Investigator List).

184B5 has also been exposed to transfection with erbB-2, mutated erbB-2, and the insulin receptor. ErbB-2 alone (**184B5-E**) made the cells capable of AIG (~5-15%, large colonies), while mutated erbB-2 additionally made them capable of tumor formation in nude mice. Overexpression of the insulin receptor also made the cells capable of some AIG. We can make available our stocks of 184B5-E.

II. C. Characterization of 184A1, 184B5, and derivatives (references: Stampfer & Bartley, 1985; Stampfer & Yaswen, 1992; Stampfer & Yaswen, 1993; Sanford et al. 1992; Lehman et al. 1993; Thompson et al, 1994; Sandhu et al, 1997; Brenner et al, 1998)

Differentiation/maturation markers: In general, 184A1 and 184B5 have a somewhat more mature phenotype than finite lifespan 184. However, it is important to recall that these lines were derived from cells grown in MM medium, which contains cells with phenotypes more mature than basal. We don't know the cell of origin of the EL 184Aa and 184Be precursors to these lines. Both lines maintain some expression of keratins 5 and 14, but at significantly decreased levels, while expression of keratin 18 is increased relative to normal post-selection 184 HMEC. Both lines have barely detectable levels of vimentin. 184B5 strongly expresses the luminal and tumor associated PEM antigens, while 184A1 has some but lower expression of PEM. The tumorigenic transformants, A1N4-TH and B5-KTu have very low levels of keratin 5 and increased levels of keratin 18. While B5-KTu remains vimentin negative, the A1N4-TH cells show re-expression of vimentin. We have not been able to detect keratin 19 or estrogen receptor in any of these lines. Thus none of these lines fully resembles the tumor cell in vivo, and the phenotypic differences between the immortal lines and MCDB 170 grown 184 HMEC may just reflect maturation states (i.e., not be related to the immortalization process). Like normal proliferative HMEC, they are keratin 19 negative.

Fibronectin represents about 10-20% of the protein secreted by normal HMEC in culture. In many transformed cells, the level of fibronectin mRNA and protein synthesis is decreased. Expression of fibronectin is greatly reduced in 184A1 and to a lesser extent in 184B5. However, we do not know if fibronectin secretion would normally be lower in HMEC with a more mature phenotype. Upregulation of fibronectin synthesis by TGFβ remains normal in both cell lines (see section V).

[Paragraph relocated]Another approach we took to characterize differences between our normal and transformed HMEC cultures was to use subtractive hybridization to identify genes expressed in the normal HMEC, but downregulated in the immortal and malignantly transformed cells. This was how we first isolated and identified CLP (see section VI. B. for more), and observed the difference in expression of keratin 5, vimentin, and fibronectin.

The normal and transformed HMEC have also been characterized with respect to both their growth patterns and their gene expression when placed on reconstituted basement membrane material derived from the Englebreth-Holm-Swarm (EHS) murine sarcoma, which has been shown to support increased differentiated functions of a variety of cell types. Normal HMEC are capable of forming three-dimensional structures with striking resemblance to endbuds in intact mammary gland tissue, whereas 184A1 displays only less developed structures and 184B5 forms only small clusters. The A1N4-TH cells show even less structure formation than 184A1 and the B5-KTu cells resemble 184B5. We have not examined the underlying basis for these differences, and suspect that alterations in cell-cell connections may be involved. E-Cadherin is expressed by all of these cells with the exception of the aggressively tumorigenic A1N4-TH cells.

Malignancy associated markers: As mentioned earlier, neither 184A1 and 184B5 shows AIG or tumorigenicity, and they retain mostly normal growth factor dependence. They do differ from normal HMEC in having some karyotypic abnormalities. 184B5 has been shown to have a 10x higher rate of mutations at the HPRT locus than normal HMEC, reduced intercellular communication, and reduced DNA repair during the G2 phase. The ability of 184A1 and 184B5 to gain AIG and to be malignantly transformed when exposed to specific oncogenes also differs from normal HMEC.

No differences in expression or regulation of the RB protein have been detected in 184A1 or 184B5. No differences in sequence or expression of p53, or p53 dependent genes, has been seen in 184A1 or 184B5 relative to normal HMEC. However, we and others have shown that the p53 expressed by the post-selection, EL, and immortally transformed cultured HMEC (but not the p53 in cultured fibroblasts from the same person) is in a conformation recognized by antibodies that recognize mutant p53, and the half-life of the p53 protein is 3-4 hrs. We do not know the functional significance of the presence of this stabilized form of p53 in these cells. I do note that the presence of stable p53 correlates exactly with the cell types which do not express p16, and wonder if there is some causal basis for this association. The absence of p16 in 184A1, as in 184Aa, is due to mutations in both p16 alleles. In 184B5, as in post-selection HMEC, the p16 promoter is methylated. The loss of p16 expression in this system (which retains normal RB) may therefore facilitate immortal transformation, but is by itself insufficient. Thus these cell lines, while immortally transformed, do not express markers of invasive or malignant transformation, and are therefore useful in studying the process of immortal transformation per se, without many other potentially confounding factors. In comparison to almost all other existing immortal lines, they are also valuable as minimally deviant immortally transformed cells. Our recent studies have focused on elucidating the immortalization specific changes (see below).

III. The Conversion Process during HMEC Immortalization (references Stampfer et al., 1997; Garbe et al., 1998, submitted; Yaswen et al., 1998 submitted; Stampfer et al., in prep., Hosobuchi & Stampfer, 1989)

III. A. Conversion of p53+/+ 184A1, and 184B5

When 184A1 and 184B5 were initially characterized back in 1982-3, we observed two growth patterns with no obvious mechanistic explanations:
(1) Although both immortal lines maintained continuous growth in mass culture following their initial emergence, slow, non-uniform growth occurred during the first 20-30 passages. As mentioned above, 184B5 first appeared as a sickly slow growing patch. It continued to grow slowly, with a gradually

increased proliferative rate, until it achieved fairly rapid growth by passage 30. Visual observation of the colonial outgrowths indicated that many cells didn't grow, or that colonies stopped growth at small sizes. Early passages of 184A1 also contained many vacuolated and non-proliferative cells. Back in the 1980's, not being able to think of a mechanistic explanation for why so many individual cells of immortal cell lines were dying, I chose to ignore this problem and only gave out higher passages of these lines, where growth was more uniform (>p30 for 184A1 and >p25 for 184B5). But this bothered me.
(2) While I could pretend to ignore the above problem, the response of these cells to the pleiotropic cytokine TGFβ was too odd to ignore, and it was this in-my-face puzzle that led me to start unraveling the conversion process (see more about TGFβ in Section V.).

We have not seen a single finite lifespan HMEC able to maintain growth in the continued presence of TGFβ, although cells that have undergone fewer population doublings (PD) in culture could undergo 5-10 additional PD before complete cessation of growth. Cells closer to senescence stopped growth within 1-2 PD. In contrast, populations of 184A1 and 184B5 which maintained growth in TGFβ could be isolated. However, the pattern of resistance to TGFβ-induced growth inhibition by these lines was unusual. 184A1 mass cultures exposed to TGFβ at passages (p) 28-35 displayed severe growth inhibition, but a small subpopulation of cells maintained active growth. Assuming these resistant cells represented rare mutations, we attempted to obtain pure populations by clonal isolation. However, like the parental uncloned population, all four clones isolated displayed a small subpopulation of cells capable of continuous growth in TGFβ. 184B5 exposed to TGFβ at p26-40 maintained good growth, but most clones isolated at p13-16 were strongly growth inhibited. One particular severely inhibited clone, B5T1, repeatedly underwent an apparent "crisis" around p30 during which almost all the cells died. The populations derived from the few surviving cells maintained growth in TGFβ. The lack of growth inhibition by TGFβ was not due to loss of the ability to respond to TGFβ. All 184A1 and 184B5 cultures showed morphologic alterations in the presence of TGFβ, and all cells tested displayed TGFβ receptors and induction by TGFβ of extracellular matrix associated proteins .

In an effort to understand (1) why so many early passage cells from immortal lines failed to maintain proliferation, and (2) how clonal isolates rapidly produced cell populations heterogeneous for growth in TGFβ, I particularly noted the association of TGFβ resistance with an indefinite lifespan in B5T1. Since the literature at the time was associating telomerase activity with an indefinite lifespan, I considered the possibility that expression of TGFβ resistance and telomerase activity might be related, and that possibly both phenotypes were not initially expressed in immortally transformed cells. Perhaps these cell lines were initially only "conditionally immortal", i.e., permissive for immortality but an additional step was required for them to obtain a uniform indefinite lifespan. To test this hypothesis, we proceeded to carefully characterize and ascertain possible associations among morphology, growth capacity in the absence and presence of TGFβ, telomerase activity, and telomere length in 184A1 and 184B5 at different passage levels. I summarize our results below and then provide more details.

Early passage 184A1 and 184B5 cells are only conditionally immortal. The cell lines, but not each individual cell, have indefinite growth potential. These early passage conditionally immortal cells express little or no telomerase activity and show no ability to maintain growth in TGFβ. The conditionally immortal cells presumably harbor a (presently unknown) mutation which permitted their continued growth past replicative senescence, but this mutation did not result in immediate expression of telomerase activity. Telomeres continue to shorten with increasing passage. Cell populations whose mean TRF (terminal restriction fragment) length had declined to < 3 kb exhibited slow heterogeneous growth and contained many non-proliferative cells. These cells also accumulated large quantities of the CKI p57, which we believe is responsible for the poor growth. Telomerase activity is first detected when the telomeres become critically short, mean TRF ~2.5-2 kb, and activity levels gradually increase thereafter. RT-PCR indicates that hTERT mRNA is not present in the early passage 184A1, but is present in fully immortal 184A1. Around the passage levels that telomerase activity can first be detected, there begins a very gradual increase in the number of cells displaying progressively increased ability to maintain growth in TGFβ (see Table 3). By the time the mean TRF has stabilized at > 3 kb, the cells have converted to full immortality, characterized by high levels of telomerase activity, uniform good growth in the absence or presence of TGFβ, and no expression of p57. We have used the term "conversion" to describe this gradual process. The consistent and reproducible manifestation of conversion by repeatedly cloned cell populations, and the very gradual nature of the conversion process, suggest an epigenetic mechanism. We postulate that there exists an inherent epigenetic mechanism to reactivate telomerase when telomere length

becomes critically short. This program is not normally encountered in human cells due to the multiple checkpoints imposed by a stringent replicative senescence mechanism to prevent proliferation of cells with shortened telomeres. Gradual, epigenetic-based changes in gene expression might occur in response to development of extremely short telomeres through alterations in heterochromatin conformation and/or altered transcriptional activity as a result of redistribution of telomere associated proteins.
NOTE: This whole process of conversion and p57 expression would not be seen in cell types where adult somatic cells do not have stringent replicative senescence/telomerase control mechanisms, i.e., all rodent cells. I think this raises serious questions about the use of rodent "model" systems for studies to understand human cellular immortalization and early stage carcinogenesis. If a system doesn't model, it's not a model system.

Now, more details and data. The conversion process is illustrated for MCDB 170 grown 184A1 in Figure 6 and Table 3. Early passage (p11) 184A1 has a mean TRF of ~5 kb, shows uniform good growth (although no growth in TGFβ) with a high colony forming efficiency (CFE). However, the CFE steadily decreases with passage, with an abrupt decrease in growth around p16, at ~3 kb mean TRF. In our lab, we refer to this as "hitting the wall" because of its relative abruptness, and the cells thereafter look kind of "smashed" (those gross looking flat vacuolated cells). Between passages 18-30 the CFE remains low, and most colonies that are still growing contain a mixture of growing and non-proliferative cells. During this period, the mean TRF declined to ≤ 2 kb with a faint signal. Low telomerase activity was first detectable around passages 24-30, and increased thereafter. After p30, mean TRF stabilized at > 3 kb, the CFE increased, and the growth displayed by individual colonies gradually became uniform. The first detection of sustained growth in TGFβ was at ~p28. This growth was exceedingly poor - but it was maintained. By p30, some cells showed OK growth in TGFβ, and after p40, most cells showed good growth in TGFβ.

Figures 6: Comparison of mean TRF length, telomerase activity, and growth ± TGFβ in 184A1 at different passage levels.
Legend for Figure 6.
Panel A: TRF length, lighter shaded ovals indicate a faint signal. Panel B: Telomerase activity, determined semi-quantitatively by comparing the levels of HMEC telomerase products generated to those generated for a constant number of 293 cells (1,000 cell equivalents). The following categories were used to designate semi-quantitative values. Note that the points are presented in a semi-log form: None = no detectable telomerase products by PhosphorImager analysis; weak = approximately 5% of telomerase activity of 293 cell control; low = approximately 10% of 293 control; medium = 25-50% of 293 control; strong = 75-100% of 293 control. Panel C: Colony forming efficiency (CFE) and labeling index (LI) in colonies. TRF length, telomerase activity, CFE and LI were determined as described in Stampfer et al., 1997.

Table 3: Growth and LI of 184A1 and 184B5 colonies at different passage levels in the absence or presence of TGFβ

			LABELING INDEX (%)				
Cell Type	Pass.#	TGFβ	<10	10-25	26-50	>50	# colonies
184A1	28	-	0	12	53	35	47
	32	-	12	16	17	55	95
	38	-	10	10	12	68	389
	44	-	0	2	7	91	272
184A1	28	+	100	0	0	0	34
	35	+	59	37	4	0	83
	44	+	3	11	11	75	85
184A1-TP	28	-	0	0	0	100	17
	28	+	12	11	22	55	42
B5Y16G	25	-	6	14	44	34	50

	31	-	0	6	0	94	51
	38	-	0	0	0	100	91
B5Y16G	25	+	71	8	13	8	189
	31	+	20	28	21	31	102
	38	+	0	0	0	100	135
B5Y16G-βR	26	-	0	0	0	100	14
	26	+	0	10	9	81	17

Legend for Table 3: Single cells were seeded and the LI ± TGFβ in colonies containing >50 cells was determined as described in Stampfer et al., 1997. # colonies refers to the number of colonies counted to determine percentage LI. 184A1-TP and B5Y16G-βR represent populations derived from isolated, early converting cells. 184A1-TP appeared in a slow growing conditionally immortal 184A1 p23 population, distinguishable by its much more rapid growth. B5Y16G-βR was derived from a rare colony that grew well in TGFβ at p24 from the B5Y16G clone of the B5Y16 clone of 184B5. The data in this table also indicate that the phenotype of uniform good growth minus TGFβ is acquired prior to that for good growth in the presence of TGFβ.

Similar overall results were seen with 184B5. However, unlike very early 184A1, early passages of 184B5 grow slowly, the mean TRF when first tested was ~3 kb, and the population already showed some heterogeneity in growth. Some cells capable of maintaining poor growth in TGFβ were already present. Given this heterogeneity, we studied clonal isolates of early passage 184B5. Clones isolated at p15 showed a large range of growth capacity. Some didn't maintain any growth after 2-3 passages, some showed heterogeneous slow growth, and some had mixed slow and faster growth. Basically, those that didn't maintain growth also showed no growth in TGFβ, no or weak telomerase activity, and short mean TRFs, < 2.0-2.5 kb, with faint or very faint signals. The clones with slow growth behaved similar to the above description of 184A1. Repeated examination of the same clones repeatedly gave the same pattern of conversion.

One clone, B5Y16, was already heterogeneous for growth ± TGFβ when first observed at p17. This extremely rapid generation of heterogeneity in a clonal isolate was further investigated by isolating subclones of B5Y16 at p20. These also unfolded the whole range of phenotypes, from no growth, 184A1-like growth, to a few clones that displayed an already fully immortal converted phenotype. B5Y16 and its subclones demonstrate that a cell population obtained after less than 10 Pd from one conditionally immortal cell may be widely heterogeneous. The B5Y16G cells, which are a subclone of a clone (B5Y16) of a clonal cell line (184B5), are a good illustration of the inherent heterogeneity in growth response to TGFβ and the gradual nature of conversion (see Figure 7 and Table 3). Although growth was slow and non-uniform when first observed at p21, by p24 rare colonies with good growth ± TGFβ were present. B5Y16G was seeded at clonal densities at p25 and examined for growth ± TGFβ. Heterogeneity was clearly visible in and among these single cell outgrowths. By p38, all B5Y16G cells gave rise to good growing colonies ± TGFβ. These data with the 184B5 clones and subclones are inconsistent with a rare mutational origin of the converted phenotype.

Figure 7: Heterogeneity of subclone B5Y16G p25 colony growth in TGFβ.
Legend for Figure 7. 1000 cells were seeded into 100 mm dishes and exposed to 5 ng/ml TGFβ 15 days after seeding. Cells remained in TGFβ an additional 18 days and were labeled with 3H-thymidine for the last 24 hrs. The Giemsa stained, single-cell derived colonies shown are from the same dish. (a) colony with no growth in TGFβ; (b) mostly flat colony with rare scattered labeled cells; (c) colony with growing small cobblestone cells amidst flatter cells with little growth; (d) colony with larger growing areas of small cobblestone cells amidst flat cells; (e) rare large colony with uniform good growth in TGFβ.

In both 184A1 and 184B5 we have observed some instances of early conversion to full immortality. In 184A1 rare early converters rapidly take over the very slow growing non-converted population (e.g., 184A1-TP). In all cases, the mean TRF of early converters when initially examined was short (2.0-2.7 kb), and there was a correlation between ability to grow in TGFβ and telomerase activity. These short mean TRFs indicate that newly converted cells arise from cells with critically short mean TRFs.

III. B. The effects of viral oncogenes on conversion of 184A1

A gradual conversion process had not been previously reported during human epithelial cell immortalization. Most reported studies of human epithelial cell transformation in culture have used viral oncogenes and/or inactivation of the p53 gene to facilitate more consistent and efficient immortalization. The overall process of immortalization described above for our chemically transformed immortal HMEC lines did not correlate with the reported descriptions of immortal transformation following viral oncogene exposure. We wondered if the potential of these viral oncogenes to simultaneously inactivate many cellular checkpoints was producing different patterns of immortalization than would occur in the generation of minimally deviant immortal cell lines with wild type p53 and RB[]. We therefore examined the consequences of exposing early passage (p12) conditionally immortal 184A1 to specific viral oncogenes: HPV16 -E6, -E7, SV40T, or Ad5 E1A.

The brief summary: Exposure to HPV16-E6 resulted in near immediate conversion to the fully immortal phenotype (good growth ± TGFβ, high telomerase activity, stabilized telomere length). Exposure to HPV16-E7 and SV40T greatly accelerated acquisition of some conversion phenotypes (uniform good growth, high telomerase activity, stabilized telomere length) and led to the immediate ability to maintain some growth in TGFβ. Ad5 E1A caused what appeared to be massive apoptosis, though some cells survived and rapidly converted. A mutated HPV16 E6 oncogene unable to inactivate p53 was still capable of near immediate conversion of p14 184A1. We conclude that the multiple activities of these viral oncogenes (inactivation of p53 and RB, and many other characterized and as yet uncharacterized additional functions) may greatly accelerate a step in HMEC immortal transformation - conversion - that might otherwise be a key rate-limiting step. The ability of these oncogenes to simultaneously inactivate many cellular checkpoints is likely responsible for their capacity to achieve reproducible immortalization of HMEC and other human cells. More details below.

[the rest of this section III.B. will not be posted until the paper is accepted for publication]

Figures 8A&B and Table 4 illustrate the effect of retroviral infection of p12 184A1 with vectors containing the indicated viral oncogenes, or the LXSN control vector.

Legend for Figures 8. Good growing 184A1 p12 were infected with retroviral vectors containing the HPV16 E6, HPV16 E7, or SV40T genes, or control LXSN-based retrovirus. Infected cells were obtained following selection in G418 for 10 days. The infected (184A1-E6, 184A1-E7, 184A1-T) and control (184A1-LXSN) cultures were then maintained in MCDB 170 medium with periodic assays for telomerase activity, mean TRF length, and growth ± TGFβ as described in Stampfer et al 1997, and the Figure 6 legend, however the scales for telomerase activity and resistance are altered compared to Fig. 6.
(8A) data for 184A1-E6
(8B) data for 184A1-E7 and 184A1-T

Table 4: Growth and LI of retrovirally infected 184A1 colonies at different passage levels in the absence or presence of TGFβ

			LABELING INDEX (%)				
Virus	Pass.#	TGFβ	<10	10-25	26-50	>50	# colonies
LXSN	13	-	0	0	0	100	203
	15	-	0	1	7	92	182
	17	-	15	26	26	33	81
	21	-	14	22	29	35	59
	23	-	1	14	43	42	91
	28	-	2	10	48	40	85
LXSN	21	+	100	0	0	0	66
	23	+	96	4	0	0	78
	28	+	100	0	0	0	39

HPV16-E6	13	-	0	0	0	100	49
	15	-	2	0	0	98	63
	17	-	0	0	0	100	253
HPV16-E6	13	+	14	11	39	36	28
	15	+	0	2	11	86	88
	17	+	0	0	0	100	79
HPV16-E6JH26	17	-	0	0	0	100	51
	17	+	0	0	26	74	53
HPV16-E7	14	-	1	0	5	94	96
	15	-	0	0	11	89	27
	17	-	34	24	42	0	42
	21	-	5	7	11	77	122
	25	-	0	12	33	55	42
	29	-	0	0	0	100	78
HPV16-E7	14	+	26	35	27	12	94
	15	+	15	51	30	4	168
	17	+	34	38	28	0	82
	21	+	22	44	29	5	57
	25	+	23	33	32	1	85
	29	+	6	31	42	21	67
SV40T	13	-	0	0	0	100	108
	14	-	1	0	2	97	92
	16	-	2	0	4	94	48
	17	-	18	14	26	42	88
	21	-	0	0	0	100	29
SV40T	13	+	12	9	31	48	52
	16	+	11	7	27	55	44
	17	+	0	11	27	62	26
	21	+	1	4	17	78	72
	27	+	0	0	0	100	132

Legend for Table 4: Single cells were seeded and the LI ± TGFβ in colonies containing >50 cells was determined as described in Stampfer et al., 1997. # colonies refers to the number of colonies counted to determine percentage LI.

The 184A1-E6 culture showed high levels of telomerase activity when first assayed at p12, and at all passages examined thereafter. Unlike the control cultures and consistent with its expression of high telomerase activity, 184A1-E6 maintained a mean TRF of ~5 kb with continued passage. 184A1-E6 maintained uniform good growth, with no evidence of slow heterogeneous growth and was already capable of good growth in TGFβ at p13. At p15, 184A1-E6 showed very low levels of p53 protein, presumably due to degradation of p53 by the ubiquitin-dependent proteolytic pathway.

We also tested the effects of several different mutated HPV16-E6 genes. Five mutants (HPV16 E6 Cys-63-Gly, Cys-63-Arg, Cys-63-Ser, Cys-106-Arg, and Trp-132-Arg; obtained from Vimla Band, Tufts U), with low or no binding to or degradation of the p53 protein, had no effect on 184A1 conversion, behaving like the vector alone control. Contrasting results were seen with the amino terminal HPV16 E6 mutant E6JH26 (obtained from Denise Galloway, U. Wash.), which has also been reported not to bind or target p53 for degradation and not to affect p53 transactivation, but which does bind the E6-associated protein E6-AP and has been shown to activate low levels of telomerase in finite lifespan human keratinocytes and mammary cells. 184A1-E6JH26 induced a similar near immediate conversion as wild-type E6, indicating that the E6 oncogene does not need the ability to inactivate p53 to induce a fully immortal phenotype.

Unlike 184A1-E6, 184A1-E7 showed no telomerase activity at p12. Low levels of activity were detected at p18 and increased thereafter. By passages 25-29, high levels of telomerase activity were present. 184A1-T exhibited an even more accelerated expression of telomerase activity. Very low levels could be detected at passages 12-15, and high levels were detected by p23. Consistent with the telomerase activity data, the mean TRF length of both 184A1-E7 and 184A1-T showed an initial decline from ~5 kb at p13 to faint signals of ~3.5 kb at p21, followed by stabilization of telomere length. Both 184A1-E7 and 184A1-T experienced some heterogeneous growth between passages 16-20 (see Table 4). 184A1-T populations showed the presence of large flat vacuolated cells similar to those observed in the 184A1 control populations during the period of slow heterogeneous growth. Many colonies with poor or non-sustained growth were visible at these passages. Uniform good growth was seen by p21 in 184A1-T and by p29 in 184A1-E7. The majority of 184A1-E7 and 184A1-T cells were able to maintain some (although not yet good uniform) growth in the presence of TGFβ at the earliest passages tested, indicating that this capacity was rapidly conferred by the expression of the viral oncogene, even before detection of telomerase activity. This result suggests that these two phenotypes of fully immortal HMEC can be acquired independently in the presence of specific viral oncogenes. The HPV16 E7 and E1A oncogenes have been reported to bind and inactivate p27, which has been associated with TGFβ growth inhibition in our HMEC system and other cell types. This additional function of the E7 oncogene may account for its ability to rapidly confer TGFβ resistance to conditionally immortal 184A1. Although a similar activity has not been reported for the SV40T oncogene, our results suggest that it too may be capable of inactivating some aspect of the TGFβ growth inhibition pathway.

Previous reports indicated that HPV16 E6 could induce low levels of telomerase activity even in finite lifespan human epithelial cells, so we examined our finite lifespan HMEC to determine whether HPV16-E6 could induce telomerase activity, and if so, whether it was dependent upon specific parameters of the HMEC population; i.e., growth conditions, age in culture, or telomere length.

Four different sets of finite lifespan 184 HMEC were examined: (1) pre-selection cells grown in MCDB 170; this population was infected at p2 when still proliferative, and assayed at p3 when it contained mostly poorly growing cells and had a mean TRF of ~8-7 kb; (2) post-selection cells grown in MCDB 170, infected and assayed at p9 and p20; both these populations had active cell division, mean TRF ~7 kb and 5.5 kb respectively; (3) EL 184Aa, the precursor of the 184A1 line, grown in MCDB 170, infected and assayed at p8 and p13; both populations had active cell division, mean TRF ~6 kb and 5.2 kb respectively; (4) 184 HMEC actively growing in the serum containing MM medium, infected at p3 and assayed at p4; mean TRF between 8-8.8 kb when assayed at passages 3-6. As expected, no telomerase activity was detected in the pre- or post-selection 184 or 184Aa without the E6 oncogene. Introduction of the E6 gene resulted in low telomerase activity in the post-selection 184 p9 and in the 184Aa p8 and p13 cells. Repeated independent infections showed no telomerase activity in the near-senescent, but still proliferating, p20 post-selection 184 cells, nor in the poorly growing pre-selection p3 cells. In contrast to the cells grown in the serum-free MCDB 170 medium, early passage 184 grown in MM showed a low level of telomerase activity, even in the absence of the E6 gene. This low telomerase activity was further increased to a medium level after the introduction of E6. These data may bear on previous reports indicating that certain populations of cultured HMEC may have low levels of telomerase activity. It is possible that the growth conditions, or selection for growth of specific breast epithelial cell types in culture, can influence whether this activity is present in the uninfected as well as E6 infected populations. These results show no strict correlation between the level of telomerase expression induced by E6 and the age in culture or mean TRF of the cell population. We saw no correlation between proliferative capacity and the expression of telomerase activity in the finite lifespan HMEC, as the HMEC population with the most long-term proliferative capacity, the post-selection cells, consistently shows no telomerase activity. However, a caution - these results are specific for specimen 184. Preliminary testing of HMEC from other specimens suggests that there could be some interindividual variability. We have not had the resources to check this further.

The rapid induction of telomerase activity in p12 184A1 by HPV16-E6, as well as its ability to induce telomerase activity in finite lifespan HMEC with mean TRF values of 8-5 kb, suggest that it acts through a mechanism other than an epigenetic turn-on of telomerase activity resulting from critically short telomeres. Telomerase activity is present in both tumor cells and cells with a high self-renewal capacity. In tumor cells and immortal cell lines, telomerase activity is associated with telomeres that are generally shorter than those found in finite lifespan cells However, telomerase positive cells with high self-renewal capacity

do not necessarily exhibit shorter telomeres. Possibly, the mechanism by which HPV16-E6 reactivates telomerase is more closely related to the regulation of telomerase activity in normal telomerase positive cells than to the reactivation of telomerase which occurs during malignant progression.

III. C. Generation of p53-/- HMEC lines 184AA2 and 184 AA3, and p53+/+ 184AA4

Our studies on conversion raised the question of what was the very rare event, presumably a mutation, responsible for the transformation of finite lifespan EL cultures into conditionally immortal cell lines. In our system, the data indicated that it was not changes in p53, RB, p16 or telomerase expression - the usually identified or postulated culprits. To address this question, we decided to try the Genetic Suppressor Element (GSE) strategy. GSEs are cDNA fragments that could potentially interfere with normal gene functions by either encoding antisense RNA or inhibitory protein fragments. Assuming that immortality represents a loss-of-function, the hope was that disruption of this normal function by a GSE would enable us to identify gene products lost during immortal transformation. A library of small cDNA sequences obtained from normal 184 HMEC was introduced into p12 184Aa (the EL precursor of 184A1) using high titer amphotrophic retrovirus. Three different new immortally transformed cell lines designated 184AA2, 184AA3, and 184AA4 resulted. Unfortunately, none of these lines demonstrated loss of the gene originally responsible for generating 184A1, and so the nature of this step is still unknown. On the up side, we did generate three new immortal HMEC lines, two of which (184AA2 and 184AA3) are p53-/-. These data are not in publication yet, but I include some generic information here since I have been distributing some of these lines already. 184AA2 and 184AA3 provide p53-/- closely related matches to the p53+/+ 184A1 and 184AA4 lines, since all are derived from the same 184Aa EL population.

PCR analysis indicated that 184AA3 had a single viral insertion, but the virus had no cDNA insert. Inverse PCR to isolate the flanking genomic DNA, and sequence analysis of the recovered clones, showed that the viral insertion was near the beginning of the first intron of p53. This was confirmed by Southern analysis of genomic DNA probed with p53 sequences. Southern analysis of 184AA2 also showed that one of the three viral inserts was in the p53 gene. Although we do not yet know the status of the remaining p53 allele, these lines show little or no expression of p53 by Western blot analysis. RT-PCR also indicates that 184AA3 has no band corresponding to a p53 transcript. Thus, these lines suggest that total loss of p53 - while not necessary - may be sufficient to immortally transform 184Aa. 184AA4 had no viral inserts and so presumably represents another spontaneous transformation from 184Aa. Like 184A1, 184B5, and post-selection 184, 184AA4 expresses a stable p53 protein. 184AA4 also undergoes a conversion process similar to 184A1.

The p53-/- 184AA2 and 184AA3 lines do not undergo the same conversion process as 184A1, 184AA4, and 184B5. In 184AA2, high telomerase activity was seen at the earliest passages tested, with a mean TRF ~4 kb and no further telomere loss. The only indication that 184AA2 was still undergoing any aspects of conversion was its initial lack of uniform good growth in TGFβ. In 184AA3, the earliest passages testable (p17) had medium telomerase activity which increased to high by p23. Initial mean TRF was ~3.5 kb and stabilized around 4 kb by p23. Early passages did not show good growth ± TGFβ, but a relatively rapid gradual increase in growth capacity led to uniform good growth minus TGFβ by ~p23 and plus TGFβ by ~p30. Neither 184AA2 or 184AA3 had detectable levels of p57 at the earliest passages tested.

These results suggest that p53 may play an additional role in transformation and tumor suppression. Absence of p53 may mean that instead of a long very gradual conversion process with slow growing cells, a more aggressively proliferative population of fully immortal cells may arise relatively quickly. We have not yet performed functional studies to determine if the absence of p53 is directly responsible for this greatly accelerated transformation to full immortality.

NOTE! NOTE! NOTE!: 184A1, 184B5, and 184AA4 are **immortally transformed** cell lines. 184AA2, and 184AA3 are **immortally transformed** cell lines that are also p53-/-. Normal human somatic cells are **never** immortal, do not express high levels of telomerase, have wild type p53 and RB, and normal karyotypes. Any somatic cell which is immortal, defective in p53 or RB, or aneuploid is **not normal.** I consider it grossly incorrect, and a potential cause of very serious scientific error, to refer to these lines, or any immortal cell line, as "normal", even if they do retain many normal properties. The defining characteristics of human cancer cells in vivo include expression of telomerase, p53 defects,

aneuploidy, and defects in the RB pathway. It's REALLY not OK to refer to cells with these characteristics as normal, or "normal" (as I sometimes see). They aren't normal, so calling them normal or "normal" is simply scientifically incorrect. Would one call a mutated p53 gene "normal"? Then why call a cell that contains only mutated p53 "normal"? This is by no means a trivial or picky point. If cells which have already acquired the defining, and potentially rate-liming steps of early stage cancer (although still not invasive) are called and thought of as normal, how are we ever going to understand the processes involved in the early stages of carcinogenic progression. Or how are we ever going to know what are normal cellular growth control processes if the cells being studied are already aberrant in their growth control properties. I believe that the common failure to recognize the important distinctions between cells that are truly normal and those which are often misrepresented as normal has allowed a serious flaw to permeate much of current "mechanistic" molecular biology in the areas of cell cycle regulation, cancer, and signal transduction.

III. D. Telomerase activity, telomere length, and growth in fully immortal 184B5

We have done just a few studies looking at fully converted HMEC populations for growth potential and telomerase activity. Uncloned 184B5 at p99, and five clones isolated at p96 were examined (Figure 9). The range of mean TRF lengths for these clones was 2.9 - 7.0 kb. To our surprise, the clone with the shortest mean TRF, B5Y9H, showed no detectable telomerase activity when first assayed at p99, although all these clones exhibited good growth ± TGFβ at that passage. TGFβ did induce morphologic changes in all five subclones. With continued passage, B5Y9H, but not the other four clones, reproducibly showed a slowdown in growth around p103, and an initial total loss of proliferation at p105. However, after a few weeks, some B5Y9H p105 cells began to give rise to large outgrowths. These cells were subcultured and have maintained good growth until at least p116. Assay for telomerase activity indicated no or very weak activity up to and including the non-proliferative p105 population. After the p105 dishes displayed the large outgrowths, telomerase activity was detectable. The mean TRF length of B5Y9H hovered around 3.0 kb prior to p105, and increased slightly thereafter. These data indicate that telomerase activity may cycle off and on even in converted cells. Unlike conditionally immortal cells, reactivation of telomerase in B5Y9H occurred relatively rapidly, within one passage. Additionally, these converted cells differed from the conditionally immortal in their ability to exhibit TGFβ resistance in the absence of telomerase activity. Their mean TRFs at the point of telomerase reactivation were also longer (~3 vs. ~2 kb).

Figure 9: Mean TRF length and telomerase activity in late passage 184B5 and subclones at different passages.
Legend for Figure 9: Assays were performed as described in Stampfer et al, 1997, and the Figure 6 legend. For TRF length, lighter shaded ovals indicate a faint signal.
(A) 184B5 and subclones;
(B) B5Y9H cells at different passage levels from two separate freezedowns. The first telomerase assay at p105 was from dishes containing vacuolated, non-growing cells. The second assay for telomerase at p105, and TRF values, were obtained from sister cultures which contained good growing patches.

III. E. Speculations about the conversion process

My style in developing this HMEC model system has been to look at large scale pictures, in low resolution, and to assist others in further investigation of interesting sub-fields at higher resolution. So my speculations about conversion, and what it all means, are more encompassing than precise. The questions that I find interesting are:
(1) What are the underlying mechanisms?
(2) Does a conversion like process occur during in vivo carcinogenesis?
(3) Can these studies lead to something clinically useful?
(4) What can this tell us about scientific approaches?

III. E. 1. Speculations about mechanisms

Inappropriate growth regulation is a hallmark of malignancy, and the loss of cellular senescence mechanisms is a defining difference between normal and tumor tissues. The vast majority of breast cancers express telomerase activity, whereas this activity is not found in normal breast tissues. Cells derived from normal human breast tissues exhibit cellular senescence in culture, whereas some cells from

tumor tissues can express an immortal potential. Most investigators would now agree that the ability to maintain replication beyond the limits set by replicative senescence is essential for cells to acquire the multiple genetic errors that permit development of invasive and metastatic tumors. Yet, despite many years of investigation by many scientists, the precise pathways by which finite lifespan human epithelial cells acquire an indefinite lifespan have not been defined. Although it has been shown that total loss of p53 and RB can facilitate immortal transformation in HMEC and other model systems, we have generated three immortally transformed HMEC lines which retain normal p53 and RB, so other pathways are possible. More importantly, the majority of human breast tumors do not contain mutations in either the p53 or RB genes, so clearly other pathways exist in vivo. What are they?

Previous models, based largely on viral oncogene mediated human cell transformation, have suggested that immortal transformation involves overcoming at least two blocks, M1 and M2. At M1, shortened telomeres signal activation of cell cycle checkpoint controls that cause a viable G1 arrest. Overcoming this block yields EL cultures, and has been ascribed to loss of normal RB and p53 function, consistent with the ability of these viral oncogenes to functionally inactivate RB and p53. The EL cells eventually reach the M2 checkpoint and undergo what has been described as a crisis, i.e., loss of proliferative capacity and death. Overcoming this block has been considered to involve a rare mutation which occurs during crisis. Since the telomeres of EL cultures continue to shorten with passage, while most immortally transformed cell lines show telomerase activity and stabilized telomere length, it has been postulated that this mutation may involve reactivation of telomerase activity.

This viral oncogene based model does not describe the process occurring in our chemical carcinogen induced immortal HMEC lines. In addition to the absence of detectable p53, RB, or telomerase mutations, the relationship among what has been called EL and crisis in the viral oncogene models, and EL and conversion in our system is not clear. Our EL cultures were defined based on their extended lifespan in a serum-containing medium, MM. We now know that these EL cells have lost expression of p16 - a process that occurs spontaneously in serum-free MCDB 170 but not in MM. Our carcinogen induced EL cultures and our post-selection normal HMEC senesce at approximately the same mean TRF length, ~5-4 kb, and show no obvious differences in the p53 or RB pathways. Both populations lack expression of p16 and have a stabilized form of wild-type p53. Some differences do exist between the EL and post-selection cells, e.g., HPV16 E6 is able to reactivate telomerase in near senescent but proliferative 184Aa EL, but was unable to do so in near senescent, proliferative post-selection 184 HMEC. We hypothesize that the EL cultures which gave rise to immortal cell lines harbor immortalization predisposing defects not present in post-selection HMEC. We do not observe an obvious "crisis" event in our carcinogen exposed HMEC immortalization system. Virtually all cells in our EL cultures senesced and died. The extremely rare conditionally immortal cells that survived then underwent a gradual conversion process which extended over a much longer time frame than has been described for crisis, with very gradual emergence of cells with progressively better growth capacity, rather than the crisis-like more rapid emergence of cells which express good uniform growth. Exposure of post-selection 184 HMEC to HPV16 E6 in our laboratory (unpublished results) has recapitulated the EL and crisis process described for immortalization by this viral oncogene. I believe that these differences between viral oncogene and carcinogen-mediated immortal transformation of HMEC suggest that caution should be exercised in generalizing among model systems.

What hypotheses about mechanisms of overcoming cellular senescence are suggested by the data from our HMEC system? First, I suspect there is more than one way for HMEC (and human epithelial cells in general) to express a senescent phenotype. Our MM grown cells lose most proliferative potential after 15-25 Pd, accompanied by increasing levels of p16 mRNA and protein, and mostly short-lived, cytoplasmically localized p53. These cells do not remain viable for months. Examination of cells from several individuals reveals a mean TRF of ~8-7 kb in these growth arrested cells. So it doesn't look to me as if they've run out of telomeres. If one defines replicative senescence as growth arrest due to telomere erosion, these cells are not expressing replicative senescence. They do express the senescence associated β-galactosidase marker. We've called this early senescence arrest (Brenner et al., 1998).

The post-selection and EL HMEC cease growth (the post-selection still remain viable for months or years) after ~50-100 PD, with mean TRF ~5-4 kb, similar to reported values for senescent human fibroblasts. I would say these cells have hit replicative senescence. They have all downregulated p16 expression by one means or another. They show no alterations in p21 or p53 expression as they senesce, and the p53 has a relatively long half-life with a nuclear localization. So many of the molecular characteristics reported for

fibroblast senescence don't seem to apply to this situation. I don't believe that this growth arrest is signaled by chromosomal damage since (1) early passage 184A1 shows only three chromosomal defects detectable by karyotyping or comparative genomic hybridization. Even after the long conversion process with extremely short telomeres, fully immortal 184A1 lineages show few or no additional detectable chromosomal changes. So immortal transformation does not require gross chromosomal instability or evidence of chromosome breakage-fusion in this system; (2) every single finite lifespan HMEC that I've observed stops growth, precisely and reproducibly, even after undergoing 50 or more PD in culture, with mean TRF ~5-4 kb. It's hard to imagine how such clockwork stoppage can arise in 100% of cells due to random chromosomal damage with increasing PD.

We suggest that escape from replicative senescence requires loss of several distinct pathways of negative growth restraints. One pathway involves regulation of RB, but loss of RB is not required. In our HMEC, loss of p16 expression seems essential for extended proliferative potential, but insufficient for immortalization. Another pathway may involve maintenance of a normal function regulated by p53, but total loss of p53 is not required. My pet theory is that very short telomeres may induce structural-tensile growth constraints which can be relieved via a loss of function change. One of the most consistent differences between finite lifespan and immortal cells is alterations in cytoskeletal structure and cell-cell interactions. The bottom line is we don't know how replicative senescence is mediated in HMEC or any human epithelial cells. Recognition of the significant differences between epithelial and fibroblast cells might help spur further research into this area.

We then suggest that alleviation of all of these senescence-associated negative growth constraints produces conditionally immortal cells which initially lack telomerase activity. We believe that reactivation of telomerase is an inherent epigenetic response to critically short telomeres. How could this be? My speculations are based on what I understand of mechanisms of telomerase control and gene expression in yeast.

1) Yeast telomeres are heterochromatic regions with large chromatin-associated protein complexes. Telomere sequence specific binding proteins recruit a complex of telomere associated proteins that may propagate the heterochromatic structure beyond the telomere region, influencing expression of nearby genes.
2) Yeast cells maintain telomere length within a narrow range. Mechanisms exist for counting the number of telomere bound proteins. Above a certain number, heterochromatic structure may prevent telomerase access and activity. Below a certain range, telomerase will have access to extend the telomere length.
3) Telomere associated proteins may bind other regions of the DNA, affecting gene transcription elsewhere. It has been proposed that telomeres can serve as reservoirs of silencing proteins.

Human telomeres are also heterochromatic regions, and human telomere binding proteins have been identified. Telomerase expressing fully immortal human cells also display relatively stable telomere lengths, and have mechanisms to maintain telomere length within a set range. The catalytic component of yeast and human telomerase are both reverse transcriptases with many functional similarities

So (remember, this is speculation), we postulate that the negative growth constraints found in cells with stringent replicative senescence mechanisms are placed on top of basically yeast-like mechanisms for regulating telomere length and telomerase activity. These constraints may have evolved as tumor-suppression mechanisms in long-lived organisms. Short lived organisms like rodents do not express stringent replicative senescence, nor stringent control on telomerase activity; loss of p16 alone is sufficient for immortalization of some rodent cells. Human cells may have retained the epigenetic mechanism to ensure telomerase activity when telomeres shorten beyond a set length. When errors allow replicative senescence to be overcome, the continued proliferation leads to continued telomere shortening which turns on this inherent response.

What I like about modeling from the yeast mechanisms is that they have the potential to explain other aspects of the conversion process. We have seen that with continued telomere shortening in conditionally immortal p53+/+ HMEC lines, there are gradual changes in cell behavior/gene expression. Drawing from the yeast model, it is possible that (1) decreasing telomere length results in heterochromatin changes, which produces altered gene expression; (2) decreasing telomere lengths results in release of telomere associated proteins which then bind elsewhere, producing altered gene expression. As consequences of

altered gene expression, a p57 mediated growth constraint is encountered, and telomerase expression is induced. We theorize that the relative levels of expression of several interacting molecules determine whether individual conditional immortal cells complete the conversion process or lose proliferative ability. External conditions may also influence an individual cell's fate; preliminary studies indicated that culture conditions (e.g., the presence of serum) influenced the efficacy with which conditionally immortal cells converted. Thus the mechanisms underlying conversion are likely to depend upon quantitative interactions of multiple cellular components, each of whose levels may vary over a continuous range. Such complex interactions may be difficult to precisely determine in a system with multiple undefined variables.

These models based on altered gene expression resulting from decreasing telomere length also have the potential to explain one of the most unusual aspects of conversion - the short time required to generate extensive heterogeneity from repeatedly cloned populations. Heterogeneity in telomere length of individual chromosomes of individual cells can be generated at each population doubling. Thus, it is theoretically possible to rapidly generate a multitude of branching lineages based upon varying telomere length. Differences in either telomere length of specific chromosomes, or the overall level of remaining telomeric repeats, could result in different gene expression among recently cloned populations.

Once cells are fully converted, they appear to have controls on telomere length similar to what has been described in yeast and the examined immortal human tumor cell lines. We postulate that malignant transformation requires additional errors providing positive growth advantages and invasive capacity. Given the relative ease with which our immortally transformed HMEC can be made anchorage independent or tumorigenic (exposure to 1 or 2 known oncogenes), vs. the extreme rarity of generating immortal HMEC lines, and the extensive period of poor heterogeneous growth in the p53+/+ conditionally immortal HMEC, we suggest that it may be the acquisition of unlimited proliferative potential which provides the rate-limiting step in malignant transformation.

III. E. 2. Speculations about conversion in vivo

I consider one of the most interesting question to be whether a conversion process occurs during carcinogenic progression in vivo. We currently have no data relevant to this, so this is just speculation. I have found one report about the existence of continued telomere shortening for around 30 passages post-establishment prior to stabilization of telomere length in two immortally transformed cell lines from breast cancer pleural effusions, and I have heard from other investigators that establishment of some human tumor cells lines in culture involves a long period of poor growth.

What excites me is that the gradual process of conversion appears to more closely model the development of human tumors, than the more rapid transformation to immortality seen in viral oncogene mediated immortal transformation. Many primary carcinomas (particularly ones that are largely p53+, like breast carcinomas) exhibit an extended period of slow, heterogeneous growth prior to the appearance of more aggressive, invasive tumors. It's possible that a gradual conversion process in vivo could, at least partially, account for this slow, heterogeneous growth. An extended period of conversion would provide a continuous pool of slowly dividing cells able to accumulate errors which both promote malignant behavior (e.g., growth factor independence, vascularization, genomic instability) and provide a selective advantage. Conversion to full immortality might not even be necessary for a tumor to become malignant and metastatic. The extended period of conditional immortality could be sufficient. Our data indicate that conditionally immortal cells can undergo a very large number of population doublings before becoming fully converted. We have also seen that there can be stochastic emergence of rare, more aggressively growing fully converted cells. Acquisition of genomic instability (which we have shown is not obligately associated with immortal transformation) would facilitate the malignant transformation of conditionally immortal cells.

Our data with the p53-/- lines raise the intriguing possibility that the poorer prognosis seen in breast tumors with mutated p53 could as least in part be related to an accelerated conversion process and absence of p57 expression.

How can any of this be translated into improvements in detection, prevention, prognostic information, or treatment of breast cancer? I don't know, and I'm not even ready to speculate on the web site. I do believe that uncovering a novel process in human epithelial cell transformation may well open the door to novel

methods for clinical intervention. I welcome input and collaboration with others on this most important topic.

III. E. 3. Speculations and opinions on how all this relates to approaches to scientific questions

CAUTION: The following presents my strong editorial opinions.

Pick up any good molecular and cell biology journal and look at the abstracts. Many make very generic conclusions about how "cells" work, e.g., cell cycle and signal transduction mechanisms, without ever identifying in the abstract what "cells" are being studied. Some never identify the "cells" in the introduction. For some papers, you have to look carefully in the Methods section to find out what cells were used. My random sampling notes that in a large percentage of these cases the "cells" being referred to are some variant of 3T3 or HeLa. Does most of the scientific community really believe that all cells are equivalent, that an immortally transformed p53-/-, aneuploid rodent fibroblast cell (3T3) or an immortally transformed functionally p53-/-. RB-/-, HPV positive, aneuploid cervical carcinoma derived cell (HeLa) will accurately reflect the cell cycle, signal transduction, etc., mechanisms of normal finite lifespan human cells? Or any normal cell? Sweeping generalizations are being presented as "truths" based on studies of cells grossly deranged in many known (and unknown) processes of growth control. Cancer cells are defined by having deranged growth control. We even have distinguished journals publishing papers that say that an immortally transformed functionally p53-/- and RB-/-, SV40T positive, aneuploid human mammary cell line, which upon continued passage can demonstrate AIG and even tumorigenicity (HBL100) are "normal diploid" cells, and can be used as "normal" controls for tumor cells. This is incontrovertibly serious scientific error. And I observe almost no notice or concern about this in the scientific community.

One cell type obviously does not accurately represent the behavior all of cell types. We are humans and not yeast; our bodies function based on very complex interactions of many different differentiated cell types - which have different histories, functions, gene expression, and fates. Our research and that of others shows significant differences in key cell cycle regulators and senescence mechanisms between normal epithelial and fibroblast cells, even from the same person's breast (e.g., p21, p16, p53, responses to TGFβ, PDGF, cAMP, HPV16). Our research shows obligate differences in cell cycle regulation between finite lifespan and immortal cells (e.g., expression and regulation of some CKIs and c-myc). Our research and others show significant differences in telomerase regulation between human and rodent cells. Yet so many biologists act as if finite lifespan and immortal, p53+ and p53-, epithelial and fibroblast, rodent and human cells, etc., are all functionally equivalent. How have we come to such an illogical, patently incorrect situation, and what can be done about it?

Let's say you're a good molecular biologist studying growth factor molecules, epidermal growth factor in particular. Would you write papers that didn't distinguish, that treated as functionally equivalent, EGF and TGF-α, amphiregulin, or VGF? EGF and the FGF or PDGF family, CSF, NGF? human EGF and the Drosophila EGF-like homologue spitz?, wild type EGF and an EGF molecule with significant amino acid substitutions? prepro-EGF and active EGF? So, as a cell biologist studying human carcinogenesis, I'm baffled as to why good molecular biologists write papers and give talks that don't distinguish, that treat as functionally equivalent: mammary, liver, colon, and keratinocyte cells; epithelial, fibroblast, macrophage, and neuronal cells; cells with wild type vs. mutant p53; human, mouse, rat, and chicken cells; finite lifespan, immortal, viral oncogene transformed, and tumor derived cells. The most logical explanation I've come up with is that the exigencies of funding have led many investigators to keep doing the next step in the same systems they've been working with, since most funding agencies are not known to fund actual proposals, and it could be scientific career suicide to do work that required more long-term system development.

In my opinion, one thing that could be done about this situation is to have journals require all papers to clearly identify the source of the cells being used, and all information potentially relevant for the conclusions drawn, e.g., finite lifespan or immortal, cell type and species, status of p53 and RB, tumorigenic properties, presence of viral oncogenes, etc. These are very significant variables, and need to be identified just as one would identifiy other variables which may significantly influence the results. Generic conclusions about "how cells work" based on studies with one or a limited number of cell types should be disallowed.

This current, to me highly illogical situation of acting as if all mammalian cell types are functionally equivalent, has led me to consider how our approaches to scientific questions influence the kinds of data we obtain, how we interpret that data, and how we value different kinds of data.

My point of view is well reflected in a quote from Dan Mazia in the ASCB newsletter: "There are many paths in the advancement of science, but the giant leaps in our Science of the Cell have been made by seeing. First we see and then we interpret and only then do we pursue mechanisms and theories. The gift of the great microscopist is the ability to think with the eyes and see with the brain".

I too believe that careful seeing is the important first step for novel scientific exploration. Thus, I see that science advances by first carefully observing nature, then discerning the patterns behind the observed phenomena, describing and measuring these patterns, and finally looking for the mechanisms which give rise to the observed patterns. The image that conveys for me this logical order is also the microscope. If one wishes to observe a structure at high resolution, one doesn't first use the highest power objective available. First the lower resolution picture is brought into focus, and then higher resolution images are gained via a stepwise approach. In this manner one has a sense of the larger context in which the observed detailed structure lies. In contrast, focusing on the detail of an area at high resolution, before ascertaining the location and function of that area within the larger context, may elucidate patterns of great beauty and elegance. These lovely patterns, however, may not provide much predictive power about the behavior and organization of the larger scale structure of interest.

So, what I see, is that many molecular biologists seem to believe that the most important thing is to explain "mechanisms" at the highest available resolution. Looking at larger scale pictures at lower resolution is commonly dismissed as "phenomenology", unworthy of serious attention or funding. In the absence of stepping back to grasp the more encompassing picture, more and more detailed studies are being done using the same cell culture technology previously employed. This cell culture technology may have been the state-of-the-art 10, 20, 30 years ago, but is now very dated. Much better options are currently available, and even better options could be developed if resources were expended in that direction. In contrast, most molecular biologists would consider it silly to even think of using 10, 20, 30 year old molecular techniques, when much better options are available. And they would consider expending resources to improve molecular technologies resaonable and valuable. I think this attitude is detrimental to the advancement of science. I believe it has led to some very elegant studies being done in cell systems that are basically uninterpretable if the question of interest is understanding normal human cellular physiology, and the derangements which transform normal human cells into malignant. How can one possibly know if results seen in deranged cells reflect normal human unless the studies have already been done on normal human cells. I see no good reason for most of the continued use of cell technology like 3T3 and HeLa for studies on topics like cell cycle regulation and signal transduction. Much better options are available, although they may take a little more effort to start.

Yes, it is somewhat more difficult to use normal human cells, and there is no easy way to do in vitro - in vivo correlations, or things like knockout experiments. Sometimes experiments need so many cells with sufficient PD that immortal lines will be required. There will still be a need for model systems that model. Nonetheless, I believe that if there were the collective agreement, bolstered by funding support, to put more resources into development of cell culture systems that can optimize modeling of in vivo reality (including ones that allow study of cell-cell and cell-matrix interactions) this could be accomplished in a reasonable time frame. I truly believe that most scientists are motivated to discover the true laws of nature and to better the human condition. While there is no way to know for sure the eventual significance of one's research on human health and well-being, I think we can greatly improve our odds by avoiding obvious anachronisms like using HeLa to study cell cycle control. If you must use immortal cells, minimally deviant lines like 184A1, 184B5, and MCF10A are available.

One more personal point of view. In biological systems, complexity and productivity are enhanced by diversity. So too, I believe the scientific endeavor is enriched by including those with a diversity of scientific approaches, and impoverished if we are all required to conform to a majority mold which does not fit. Since some of my style and skills differ from what I perceive as the accepted and valued current norm, I am sensitive to how minority viewpoints and skills get excluded. I survived many years of being told that what I was doing (trying to develop improved human cell culture technology to better address

questions of human cell physiology and carcinogenesis) was just phenomenology, "gardening", even "witchcraft". Valid, although minority approaches can illuminate things that others miss, can create resources that others can't. While I think it appropriate for most scientists to follow the "high resolution - small field" approach, I believe we all benefit if our scientific culture (including funding and publishing organs) has the tolerance limits both to openly support some who follow the "low resolution - large field" approach and to direct "high resolution - small field" studies to areas that appear most promising based upon the "larger field" work.

IV. Synchronization of HMEC Cultures and Role of EGF Receptor Signal Transduction (references: Stampfer, Pan et al. 1993; Stampfer and Yaswen, 1993; Bates et al., 1990)

One of the my long-tern goals in developing the HMEC system was to ask questions related to mechanisms of growth control, such as those controlling expression of finite lifespan, senescence, escape from senescence (immortality), and the role of specific positive and negative growth factors in normal and transformed cells. I assumed that these processes would be connected to cell cycle control, and that in order to examine the cell cycle, it would be necessary to obtain synchronized cell populations. Therefore, I looked for a method to synchronize the HMEC. Additionally, I wanted to find a method that would not involve use of metabolic inhibitors or general starvation, and thus potentially not be cytotoxic or stress inducing.

We had previously shown a stringent requirement for EGF receptor (EGFR) ligands (e.g., EGF/TGFα) for clonal growth of the HMEC, although the normal HMEC could grow in mass culture without addition of exogenous EGF. Further study demonstrated that the mass culture growth was due to an autocrine loop resulting from endogenous production of EGF-like ligands such as TGFα and amphiregulin. Blockage of EGFR signal transduction with an antibody to the EGFR prevented growth. Although 184A1 and 184B5 synthesized similar amounts of TGFα as normal 184 HMEC, they failed to secrete this protein, and thus required addition of EGF to the medium for growth (see figure 11).

The above data suggested that blockage of EGFR signal transduction might provide a method to reversibly arrest these HMEC, and this possibility was examined in detail. After growth with EGF to midconfluence, HMEC cultures were maintained for 48 hrs in medium without EGF and containing monoclonal antibody (MAb) 225 to the EGFR. Cells were then refed with medium containing 5 times normal EGF. During exposure to MAb 225, the HMEC acquired a less refractile morphology with increased cell-cell contact, decreased motility, and few mitoses. After re-exposure to EGF, the typical cobblestone epithelial morphology and many mitoses were visible by 24 hrs. Normal HMEC could be maintained for at least 18 days in EGF deficient medium plus MAb 225 and still regain a normal cobblestone appearance with many mitoses after EGF re-exposure, suggesting that the growth inhibited cells were arrested in a viable, non-cytotoxic state (I refer this state as sleeping/hibernating). We have not systematically tested 184A1 and 184B5 for long-term viability in minus EGF medium. Although they enter quiescence after 48 hrs without EGF, differences in the G0 state between finite lifespan and fully immortal HMEC may mean that the immortal cells differ from the normal HMEC in their ability to remain viable in "hibernation".

Protein and DNA synthesis in normal 184 and 184B5 were assayed by incorporation of 14C-leucine and 3H-thymidine (Figure 10 A&B). Protein synthesis remained depressed as long as the antibody was present and increased rapidly following re-exposure to EGF. DNA synthesis decreased 12 hr after antibody addition, and was sharply decreased by 24 hr. DNA synthesis resumed only 10 hr after EGF re-exposure and then increased sharply to a peak around 18 hr. We have examined cells from reduction mammoplasty specimens 48 and 161, and found similar results. For specimen 48, DNA synthesis following restimulation with EGF began and peaked about two hours earlier, and there was greater synchrony exiting S phase, suggesting that good synchrony could be maintained into the next cell cycle.

Figure 10. Effects of blockage of EGF receptor signal transduction on DNA and protein synthesis by normal 184 HMEC and 184B5.
(A) Cells from specimen 184 were grown in 35mm dishes in complete MCDB 170 until midconfluence. Treated cultures were then exposed to MCDB 170 minus EGF plus 8 ug/ml MAb 225 for 49 hrs, while control cultures received complete MCDB 170. After 49 hrs, all dishes were washed once with PBS and refed. Treated cultures were refed with either complete MCDB 170 containing 25 ng/ml EGF (▲) or maintained in MCDB 170 minus EGF plus 8 ug/ml MAb225 (■). Control cultures (●) were refed with

complete MCDB 170 containing 25 ng/ml EGF. Cells were exposed to a 2 hr pulse of 5 uCi 3H-thymidine (closed symbols) and 80 nCi 14C-leucine (open symbols) in 1.5 ml of medium for 1 hr before and after the indicated times. Total acid-insoluble counts were then determined and are presented on a per dish basis. (B) Cell from 184B5 were treated as for 184 with two differences: no MAb 225 was used and the cells were kept -EGF for 48 hrs.

Figure 11 TGFα production and secretion, and effects of EGF on growth and DNA synthesis of normal, immortally transformed, and EGF independent variant HMEC.
To determine the effect of EGF on growth rates, 0.5 x 10^5 cells were seeded into 35mm dishes in either complete MCDB 170, MCDB 170 minus EGF, or MCDB 170 minus EGF containing 6μg/ml of MAb 225. The number of attached cells was determined 16-24 hr later. When control cultures (complete MCDB 170) were just confluent, all cell cultures were trypsinized and cell numbers determined by Coulter Counter. To determine the effect of EGF on DNA synthesis, midconfluent cultures that had been grown in complete medium were switched to the indicated medium for 24 hr. For the last 2 hr, cells were exposed to 4 μCi ^{3}H-thymidine in 1.5 ml. Acid precipitable counts were determined by scintillation counting. To determine TGFα synthesis and secretion, cells were grown in 60mm dishes until subconfluence. 24 hr conditioned medium was then removed, and the cells harvested and frozen. Radioimmunoassays of medium and cells were performed by Robert Coffey, Vanderbilt University. These data indicate that 184A1 and 184B5 synthesized amounts of TGFα similar to normal 184 HMEC, but failed to secrete this protein. The ability of the variant lines to maintain growth in the absence of EGF did not appear to be due to increased TGFα secretion. Although A1NE and B5NE could continue to grow in the absence of EGF, their rate of growth was decreased, and they were still sensitive to MAb 225 induced inhibition of growth and DNA synthesis. It is possible that increased synthesis of another ligand for the EGF receptor, such as amphiregulin, may be responsible for their altered growth properties. The ENU induced variant, A1ZNEB also grew faster in the presence of EGF, but it was no longer sensitive to MAb 225 inhibition. Additionally, it showed significantly increased levels of cell-associated TGFα protein. One possible explanation for this phenotype is internal or membrane associated stimulation of the EGF receptor. The ENU induced variant B5ZNEI showed no difference in growth rate with or without EGF, but was still partially sensitive to MAb 225 inhibition.

Specimen 184 was tested for how long a period of EGF re-exposure was needed following arrest to allow cells to subsequently enter S phase, with the result that a 1 hr exposure was sufficient to allow the majority of cells capable of cycling to later enter S. Thus EGF seems necessary just to get the cells into cycle (a competence rather than a progression factor). All the other growth factors were present in the medium, so we can not say if any of them specifically function as progression factors.

The above results suggested that HMEC restimulated with EGF following the growth arrest were exiting a G_0 state and entering S phase in a highly synchronous fashion. Examination of early response gene expression supported this conclusion. Expression of c-myc, c-jun and c-fos was readily detectable in normal cycling HMEC cells, but decreased during growth arrest. High levels of mRNA for all these genes were observed at 1 hr following re-exposure to EGF. 184B5 differed from normal HMEC in not showing any decrease in expression of the early response genes during the G_0 arrest, while 184A1 at p31 showed a partial decrease. We now know that this difference correlates with conversion to full immortality. Late passage 184A1 as well as late passage 184B5 do not downregulate c-myc mRNA or protein during the G_0 state. Synthesis of TGFα mRNA, which was also inhibited in the presence of MAb 225, was detected by 2 hr after EGF re-exposure. Some mRNA species, such as for keratin 5 and CLP, continued to be expressed during the growth arrest.

Studies done largely with growth arrested fibroblast cells have defined a G_0 state characterized by low metabolic activity, a rapid increase in levels of mRNAs for certain early response genes upon release from the growth arrest, and an increase of 6-7 hr in the time required to begin DNA synthesis following release from growth arrest, relative to continuously cycling cells. These properties are all observed with the HMEC growth arrested by EGFR blockage, suggesting that this is a G_0 arrest. Thus, blockage of EGFR signal transduction is sufficient by itself to cause normal and immortally transformed HMEC to enter a G_0-like resting state. Unlike fibroblast cells from the same specimens, the levels of c-myc mRNA in HMEC remained high throughout the cell cycle, while c-fos and c-jun mRNA, though showing cycle dependent fluctuation, were readily detectable in the cycling epithelial cell populations.

Exit from a G_0 state is not the same as passage from M into G1. In experiments with 184B5, we examined the mRNA synthesis into the second cycle, and could see an absence of the strong burst of early response gene expression as cells entered G1 without a G_0 exit. Levels of these mRNA species did increase, and it could be seen that the rise in c-fos expression preceded that for c-myc and c-jun. To study the cycle without a G_0 arrest, one can follow the cells into the second cycle, or use a method for G1 arrest developed by Keysomarsi et al., 1990, which utilizes Lovastatin, an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase.

V. TGFβ Effects on Normal and Transformed HMEC (references: Hosobuchi & Stampfer, 1989; Stampfer, Yaswen et al. 1993; Slingerland et al, 1994; Sandhu et al, 1997; Stampfer et al., 1997)

TGFβ is a pleotropic cytokine known to be a potent growth inhibitor of normal epithelial cells in vitro and in vivo. It modulates several key physiologic processes such as wound healing, differentiation, and tissue morphogenesis and remodeling, and is also thought to be involved in carcinogenic progression. Varying degrees of resistance to TGFβ induced growth inhibition are seen in human carcinoma cells, and this loss of negative growth regulation may contribute to tumor development. While in some instances, resistance correlates with loss of functional type I and II TGFβ receptors, most resistant tumor lines express normal numbers of apparently functional receptors.

I initially examined the effects of TGFβ on HMEC to determine if it could be a good method for cell synchronization. This was clearly not the case. However, in the course of these studies I found that growth responses to TGFβwere one of the clearest differences between the finite lifespan and immortal cells, and got hooked on trying to understand why (see Section III).

My first difficulty with TGFβ was simply getting consistent results on the extent and speed of growth inhibition of normal HMEC from the same individual. This mystery was partially solved when I controlled for passage level and selection batch. The effect of TGFβ on some individual specimens depended upon age in vitro. While **every** finite lifespan HMEC that we have tested is ultimately growth inhibited by TGFβ, younger cells in culture may undergo 8 or more PD before full arrest, whereas older cells stop growth in 1-2 PD , and with lower TGFβ concentrations (see figure 12). The normal HMEC show distinctive morphologic changes in the presence of TGFβ, characterized by an elongated, flattened appearance. The growth inhibition was only partially reversible, the extent of reversibility decreasing with age in vitro, and was relatively asynchronous (see figure13A). The cells were not in a resting state, since 14C-leucine incorporation indicated that protein synthesis was stimulated even as growth was inhibited (see figure13A). The growth arrest is in mid to late G1; TGFβ added at ≥ 10 hrs following G_0 exit was not growth inhibitory.

Figure 12. Effect of TGFβ on growth of normal and transformed HMEC. Cultures were seeded into triplicate 35 mm dishes (4-5 x 10-4 cells/dish) in the indicated concentration of human recombinant TGFβ1. The number of attached cells was determined 4-16 hr later. When control cultures were subconfluent or just confluent, all cell cultures were trypsinized and cell numbers determined by Coulter Counter. The attached cell number was subtracted from the final cell count. Data are presented as percentage cell number of the TGFβ exposed cells relative to the non-TGFβ treated controls. The β–resistant 184B5 were assayed at p26; the B5T1 p16 represent a very β-sensitive clone, isolated at p15, which ceased almost all growth by p30. The β-resistant B5T1 p35 represent populations derived from the few surviving cells. 184A1 was assayed at p39; the A1L5-S represent one of four clones isolated from 184A1 at p29-34, and was assayed at p32. All 4 clones gave results similar to the uncloned population when assayed within 11 passages from isolation. **NOTE**: When I refer to the finite lifespan cells as TGFβ sensitive, this means that no cell (as in zero) has been capable of maintaining growth in the presence of TGFβ. This is very different from what may be called 'TGFβ sensitive" breast tumor cell lines, where what it referred to is often a reduction in cell number or growth rate. When I refer to immortally transformed cells as TGFβ resistant, this means that the cells can maintain growth indefinitely in the presence of TGFβ. However, there may still be some reduction in growth rate or cells which don't maintain growth.

Another mystery which I never solved (or published, since I didn't know what to make of it) was stumbling across the observation that addition of MAb 225 to normal HMEC growth arrested by TGFβ led to synchronous entry into S phase, within 3 hrs, of the cell population that was reversibly inhibited (see

figure 13 A&B). This seemed to imply that some function of the EGFR was required to maintain TGFβ growth arrest in late G1.

Figure 13. Effect of addition of MAb 225 on cells arrested in late G1 by TGFβ.
(A) [in text](B) 184 p13 were seeded into triplicate 35 mm dishes and grown in MCDB 170 until sparse-midconfluent. Treated cultures were then exposed to 20 ng/ml TGFβ for 48 hr. All dishes were then washed 1X and refed as indicated. For 1 hr before and after the indicated times, cells were exposed to 5 μc 3H-thymidine in 1.5 ml. Total acid-insoluble counts were determined and presented on a per dish basis. Cells exposed to continuous TGFβ had 1/3 the cell number as control dishes after 72 hr of TGFβ exposure. TAb 1 is an antibody to TGFα. It's effect was generally similar to that of MAb 225, indicating that the observed result with MAb 225 is not due to an agonist effect; however, since EGFR ligands in addition to TGFα may be present, TAb 1 did not have as stringent an effect as MAb 225.

Examination of 184A1 and 184B5 for their responses to TGFβ is what really caught my attention. Whereas I had never seen a single finite lifespan HMEC maintain growth in TGFβ, the immortally transformed HMEC lines could give rise to populations that maintained growth indefinitely in the presence of TGFβ. The data illustrating these initially puzzling results that led to the studies described in Section III. A. is shown in Figure 12.

Although growth responses to TGFβ varied among the normal and immortalized HMEC, all of these HMEC showed a similar profile of TGFβ1 receptors and all expressed specialized responses to TGFβ including strong induction of: mRNA and/or protein for extracellular matrix associated proteins such as fibronectin, collagen IV, and laminin; the proteases, type IV collagenase and urokinase type plasminogen activator; the protease inhibitor, plasminogen activator inhibitor 1. The level of overall protein synthesis, especially secreted proteins, was increased following TGFβ exposure even where cell growth was inhibited. These results indicated that the effects of TGFβ on HMEC proliferation could be dissociated from its effects on specialized responses likely to play a role in glandular remodeling, homeostasis and/or wound healing. It should be noted that fibroblast cells can show similar specialized responses to TGFβ in the absence of growth inhibition. Fibroblasts from specimen 184 show a slight growth stimulation in TGFβ.

We next examined the effects of TGFβ on cell cycle related genes and proteins. Finite lifespan and immortal 184A1 and 184B5 HMEC populations were analyzed after exit from G0 following release from EGFR blockage. The following summarizes some of our results (some of which we have never gotten around to publishing).
1) RB is normally phosphorylated in these HMEC 5-10 hr after G0 release. Exposure to TGFβ prevented this phosphorylation in the TGFβ growth inhibited finite lifespan and conditionally immortal cells, but not in the TGFβ resistant fully immortal cells.
2) In finite lifespan and conditionally immortal HMEC, levels of c-myc, c-fos, and c-jun mRNA, and c-myc protein (c-fos and c-jun proteins were not examined) are low during G0 arrest. In contrast, fully immortal 184A1 and 184B5 did not show downregulated expression of these molecules during G0. TGFβ had no effect in any of the HMEC (growth inhibited or not) on the normally high level expression of these molecules seen 1 hr after G0 release. In the presence of TGFβ, normal 184 and all 184B5 HMEC examined (conditional and fully immortal) showed decreased myc levels ≥5 hr after G0 release, while levels in all 184A1 examined were not significantly affected. Thus, contrary to reports in other systems, we did not see any TGFβ effects on myc expression that correlated with extent of growth inhibition. This difference may be due to our use of cells that are all responsive to TGFβ, in contrast to studies where the cells were not only resistant to TGFβ growth inhibition, but also non-responsive. Because TGFβ can affect more than one cellular pathway, these results caution against assuming that any differences observed between responsive and non-responsive cells are necessarily contributing to the growth inhibition pathway.
3) In general, cyclins D1, D2, E, A, and B are expressed during the cell cycle with kinetics similar to reports in other cell types. Exposure to TGFβ reduces expression of cyclin A mRNA and protein in the growth inhibited cells, and has little if any effect on D1, D2, E, or cdk2 bound E, with the exception of reduction in cyclin E mRNA. cdk2 and cdk4 protein levels are constant throughout G0 and the cell cycle, and are unaffected by TGFβ in any of the HMEC.

The above studies had not detected major TGFβ induced effects on expression of cell cycle associated molecules to account for the failure to phosphorylate RB in the growth inhibited populations. In collaboration with Joyce Slingerland, U.Toronto, this led us to examine the activities responsible for RB phosphorylation, namely the cyclin/cdk complexes D/cdk2,4,6, E/cdk2, and A/cdk2, and further, the molecules capable of inhibiting this activity - the CKI.

Cyclin D1-cdk4 (or D1-cdk6) complex formation is normally detected 6-18 hr following G0 release. In the presence of TGFβ, the growth inhibited cells failed to show this complex formation, or D-associated kinase activity, whereas these complexes were unaffected by TGFβ in the resistant cells. Assays for cyclin E and A associated kinase activity also showed significant inhibition in the TGFβ sensitive cells upon exposure to TGFβ. This decreased activity was due to the presence the CKI p27. p27 inhibitory activity is normally highest in G0, and then decreases as the cells progress through G1. In TGFβ exposed normal HMEC, this activity remains elevated throughout G1. We also observed that the fully immortal cells had significantly less CKI activity in G0, independent of TGFβ exposure.

Further studies examined the role of the CKI p15. In all examined HMEC, p15 mRNA is expressed in G0 and decreases as cells progress into G1. Consistent with the report that TGFβ increased p15 mRNA levels, we observed that TGFβ caused p15 mRNA expression to remain at the G0 level even after exit into G1. However (like the studies above with c-myc), this was true for both the growth inhibited and resistant cells, indicating that this effect is not correlated with the role of TGFβ in growth inhibition. We did observe that p15 protein levels accumulated in TGFβ exposed growth inhibited, but not resistant HMEC. Measurement of p15 protein half-life showed it to be more stable in the TGFβ exposed growth inhibited cells (≥34 vs. 8.5 hr). We hypothesize that the observed increased binding of p15 to cdk4 and cdk6 in these cells stabilizes the p15 protein. This binding correlated with loss of D1, p27 and p21 associated cdk4/6 and increased p27 association with E/cdk2 complexes.

We did not detect any inherent differences in the p15 protein in the inhibited vs. resistant cells. p15 isolated from either could displace in vitro D1, p27 and p21 from cdk4 complexes isolated from inhibited cells. However, neither p15 could disrupt these preformed complexes from resistant cell lysates. It is therefore most likely that the p15 differences in the inhibited vs. resistant cells are secondary to a change in the D1/cdk4-6/p27/p21 complexes which alters the ability of p15 to bind the cdk. It is also possible that an alteration in p27 increases its affinity for cdk2 vs. cdk4-6.

V. Other Properties of HMEC System

Of the variety of studies we and others have done on these HMEC cultures, I want to include two topics in this review that, at various times, have been the subject of extensive investigation, and may be relevant to work of others.

V.A. Metabolism of Chemical Carcinogens (references: Stampfer et al., 1981; Bartley at al., 1982; Bartley and Stampfer, 1985, Leadon et al., 1988)

Before we began the studies on BaP transformation of normal HMEC, we had performed extensive studies on the capacity of human mammary epithelial and fibroblastic cells to metabolize the PAH class of procarcinogens. PAH like BaP require a series of metabolic steps for conversion of the inactive procarcinogen into the active, ultimate carcinogenic form, the diol-epoxide, which is capable of forming bulky adducts with DNA. Since the extent and pattern of BaP metabolism can vary greatly among species, as well as among different individuals and cell type within one specie, we examined the rate and path of BaP metabolism in cells from many individuals.

Our results indicated that HMEC are extremely active in metabolizing BaP through the pathways that lead to the 7,8-diol-9,10-epoxide, which can form adducts with the deoxyguanosine of DNA. in contrast, the same concentration of BaP given to fibroblast cells from the same person's breast tissue yielded a much slower rate of metabolism, mainly through pathways that do not lead to the diol-epoxide, and a much lower production of DNA adducts. At the time these studies were performed, it was surprising to observe this high degree of PAH metabolism by non-liver tissues. Whether this has any bearing on in vivo transformation of HMEC is still unknown. Of possible note is the fact that the breast consists largely of adipose tissue, in which the lipid soluble PAH can concentrate. Comparisons of BaP metabolite products

from 22 different specimen donors showed around a 5-fold range in values. Thus, this is a situation where individual variability needs to be considered. Additionally, we found that culture conditions can significantly influence the metabolites formed. The pattern of metabolites could vary with medium used (MM vs. MCDB 170), passage level (pre- or post-selection MCDB 170 cells) and use of sub-optimal culture conditions (confluent or overly acidic cell cultures).

We also showed that the damage resulting from BaP metabolism may be due to oxidative damage as well as bulky adduct formation. We found that the lethal effect of BaP correlated with the extent of thymine glycol formation (a measure of oxidative damage) rather than bulky adduct formation, and could be reduced by agents which protect against oxidative damage, such as superoxide dismutase.

V.B. Calmodulin-Like Protein (references, Yaswen et al., 1990; Yaswen et al., 1992; Edman et al., 1995)

One approach we took to characterize the differences between our normal and transformed HMEC cultures was to use subtractive hybridization to identify genes that are expressed in the normal HMEC and are downregulated in the immortal and malignantly transformed cells. Subtractive hybridization was performed between the normal 184 cell cDNA and both 184B5 and B5KTu cell mRNA. In addition to identifying fibronectin, keratin 5, and vimentin, a 350 base pair cDNA fragment was isolated which initially showed no similarity to any sequence reported in GenBank. This cDNA hybridized specifically to a 1.4 kb mRNA, designated NB-1, which was expressed in normal HMEC, but was downregulated (184B5) or undetectable (184A1) in the transformed cell lines. Sequence analysis of a full length NB-1 clone revealed a 447 bp open reading frame with extensive similarity (70%, 71%, and 80%) at the nucleic acid level to the three known human genes coding for the ubiquitous calcium binding protein, calmodulin. The similarity between the translated amino acid sequence of NB-1 and human calmodulin was 85% over the length of the entire protein.

Using Northern and PCR analysis, NB-1 mRNA has been thus far found only in normal epithelial cells and tissues from human breast, prostate, cervix, and skin. It has not been found in normal epithelial cells other than those from stratified or pseudo-stratified tissues. It was not detectable in non-epithelial cells and tissues, nor in any of the mammary epithelial tumor cell lines which we have examined. Human breast cells obtained from lactational fluids were also negative for NB-1 expression by PCR analysis.

Expression of NB-1 mRNA is not significantly decreased when cells are growth arrested by exposure to anti-EGF receptor antibodies or in senescing cells where proliferation is minimal. It is increased in cells growth arrested by TGFβ and reduced when HMEC are grown on reconstituted extracellular matrix material.

Using antisera which displayed a strong preference for NB-1 protein over calmodulin., the level of endogenous NB-1 protein in 184 HMEC was approximately 100-200 ng / 10^6 cells, a level similar to the estimated level of calmodulin in other cultured cell lines. The relative abundance of the 16 kD NB-1 protein (named calmodulin like protein, or CLP) reflected relative NB-1 mRNA levels in various cell types. In contrast, levels of calmodulin protein were nearly constant in the same cell extracts.

Using indirect immunoflourescence, CLP was shown to be present diffusely throughout the cytoplasm and, to varying degrees, in the nuclei of 184 interphase cells. During mitosis, CLP was particularly bright in regions around mitotic spindles. In 184B5, CLP expression was heterogeneous both among different cells and within individual cells; no significant CLP immunofluorescence was observed in 184A1. In surgical specimens from histologically normal breast tissues, CLP staining was strong in the majority of basal cells from small ducts. Luminal cells in the small ducts showed some staining, although not as intense. In larger ducts, staining was mainly confined to the basal cells and was generally less intense than in the small ducts. In all cases, distribution of the protein appeared uniformly intracellular. No staining was evident in basement membrane or stromal areas. In contrast to normal breast tissue, sections from six infiltrating ductal breast carcinomas were consistently negative for CLP expression. Serial sections of the normal and tumor tissues all showed abundant calmodulin expression.

CLP distribution has also been examined in other stratified and pseudo-stratified epithelial tissue sections. In normal prostate, nearly all the epithelial cells were stained to a similar degree. In normal cervix and skin, no staining was observed in the basal cell layer. In the cervix, suprabasal cells were intensely CLP

positive, with the degree of staining diminishing in the more distant upper layers. In the skin, the intensity of staining increased from the suprabasal layer until the stratum corneum, which itself was not stained. Thus, in the four different tissues examined, CLP showed distinct patterns of expression. These results suggest that the role of CLP may be defined by the differentiated state of the cells where it is expressed.

An unusual feature of CLP genomic DNA is the absence of introns, whereas all vertebrate calmodulin genes studied to date contain five similarly placed introns. It is possible that CLP may be a rare example of an expressed retroposon.

External calcium concentration has been shown to affect the proliferative potential and differentiated states of some cultured epithelial cells, including keratinocytes and mammary epithelial cells. In normal keratinocytes, increasing calcium concentrations can lead to cessation of proliferation and expression of markers of terminal differentiation, and loss of response to the calcium induced differentiation signal has been shown to correlate with the early stages of transformation. The downregulation of CLP expression observed after in vitro and in vivo transformation of HMEC may reflect a consequence of, or a requirement of the transformed state. Possibly, a particular state of differentiation is required for transformation to occur, or the transformed state may be incompatible with high expression of CLP.

VI. Information on HMEC Computer Records, Mailing Sheets, and Distribution

In order to accurately record both the many varieties of HMEC being used in my lab, and the cell cultures distributed to others, it became acutely necessary to develop appropriate record keeping practices. These have been threefold: (1) A complex relational database for recording frozen cell culture inventories and information; (2) A simple database for recording cell cultures distributed to other laboratories and a newsletter and other informational material on cell usage to be distributed along with the cells; (3) Standardized record keeping formats for my lab. More details are presented below.

VI. A. Cell Inventory Database

When I first starting collecting and freezing cell material in the days before personal computers, records were maintained on index cards on rolodexes. This was obviously not an ideal format, particularly as the number and complexity of cell types increased. We acquired a PC in 1985 and I worked with a programmer to develop a suitable inventory database using DBase. Since I am not familiar with computer programming, DBase on a PC was not an optimal situation for me. As soon as the Macintosh II and the 4th Dimension database were available in 1987, the existing program and records were transferred and refined. I worked closely with a computer programmer to design the 4th Dimension program, which is flexible enough to allow me to make adjustments in layout, data organization, and ways to select and present data. More recently, we have been able to have a computer programmer make major updates and improvements to this program.

The database consists of two main related files. One file (Inventory) records the complete identity of each frozen cell batch (identified by Specimen ID, Cell, Tissue, Passage #, and, where relevant, also Type, Subtype, FreezeDown Symbol and Selection), the number of ampoules of that batch which were made and which remain, the location of that batch of ampoules in the freezer, and a space for comments. Whenever cells are frozen, a test ampoule is included. When the test (or the first ampoule of that batch) is removed it is scored for viability, health, and the number of days it takes to reach confluence. This information is then entered into the Inventory file (unfortunately, not all batches have been tested, so sometimes I end up sending cells whose viability has not been ascertained). All freeze downs are also tested for mycoplasma contamination by Hoechst staining, and the results of this testing are entered into the Inventory file. This program allows for easy selection and sorting on any field.

The second file (Location) records the location of each individual ampoule. When an ampoule is removed, it records and files the information on date, purpose for removal, and who removed them. It also updates the Inventory file records to indicate the reduced number of ampoules remaining.

In both files, all ampoules are identified by a 5 digit code, the **FreezeDownNumber (FDN)** you see on the Mailing sheets I send. The purpose of this code is to encapsulate all of the information above into 5 digits that can be easily written on an ampoule and stored in a computer. **IT DOES NOT BY ITSELF**

IDENTIFY THE CELLS. PLEASE, DO NOT REFER TO THE CELLS BY THIS CODE! I have no idea what cells you're talking about without checking the database. These code numbers have even crept into publications - a truly confusing situation. The cells should be referred to by their Specimen Identification, Type, and Subtype (sent to you on the mailing sheets, see below).

VI. B. Cell Distribution Database

I use simple databases I created in Panorama II to keep track of cells sent to other investigators, (Recipients file) and to keep addresses and information for the Newsletter (Newsletter file). The Recipients file generates the mailing sheets that go along with the distributed HMEC. These databases allow for easy selection on all fields. Figure 14 gives an example of these mailing sheets and explanations of the categories.

Figure 14. Example of a HMEC Mailing sheet.

I started the Newsletters as the number of collaborations and those requesting cells increased. Basically, I wanted to ensure that a certain level of information about the cells was given to each investigator. Additionally, I thought it might be helpful for all those using the same cell system to be aware of what others labs were doing. Output of the Newsletters was rather sporadic. One of the goals of this website is to replace the Newsletters. Thus, this review is intended to provide the basic information (and more) that was in the newsletters and in reviews that I would send to investigators requesting cells. Additionally, methods for use of the cells (see **Procedures**) and the list of other investigators and their research subjects is available (see **HMEC Investigators List**).

VI. C. Cell Distribution

Given the large numbers and types of cells available, I prefer to talk individually with each person desiring cell cultures to determine what is most appropriate for their needs. If you want cells, you will be asked to send a brief (1 page) letter describing your planned experiments, and indicating that (1) you will keep me informed of results or major changes in planned experiments; (2) you will not give the cells to others without my permission. Let me know in you want to be on the Investigators List, and if so, include all the information as you would want it listed (including EMail address). There are also legal forms from the University of California for you and your institution to sign and return. I will **require** from you a FedEx number or equivalent to charge the costs of shipping the cells. For shipments outside the US, you will need to ensure that all proper customs forms and delivery arrangements are made. We prefer to send out frozen cell cultures; these are placed in dry ice and send overnight. We can also send live cells at room temperature if absolutely necessary, but we seriously discourage this alternative as it is much more work for us.. A mailing sheet will be sent with the cells (see above). We have not tested every freezedown (particularly the more obscure) so occasionally we will be sending cells that have not been tested for viability. I will note this on the mailing sheet. Otherwise, the cells that are being sent are known to be capable of good growth under the appropriate culture conditions.

General Cell Culture Reminders:

Taking care of normal human epithelial cells in culture bears some resemblance to taking care of children - the cells may behave by their own logic and timing, which may not coincide with that of the care provider. To optimize the accuracy and consistency of experimental results, the cell's needs have to come first. Some of the important things to remember for these HMEC:
(1) pH must be carefully controlled. The color of the pH indicator should be around salmon-orange. Yellow indicates too acid conditions; in my experience cells left at such acidity become irreversibly sick. The HMEC quickly acidify the culture medium, particularly when near confluent. We change the medium every 2 days (3 on weekends) and refeed a culture 24 hrs before subculture or experimental usage. Your results may differ if you use cells that are acidic or haven't been fed in a while.
(2) The cells do not stay healthily once they become confluent. They should be subcultured when subconfluent or just confluent. We use subconfluent cultures for most biochemical and molecular studies. Your results may differ if you use confluent (non-proliferating) cultures.
(3) Some cell biology changes as a function of age in culture. It is best to repeat experiments using cells at around the same passage level, with the same life expectancy. This is also a good practice for the cell

lines, which may change over extended periods of time in culture. Your results may differ if you use cells at very different passage levels.
(4) It's really helpful to look at the cells frequently, to become familiar with how they appear under different circumstances. An enormous amount of useful information can be gleaned simply by careful visual observation. If something doesn't look right, it probably isn't, and should be investigated immediately.

ABBREVIATIONS:

BaP: benzo(a)pyrene
CFE: colony forming efficiancy
cdk: cyclin dependent kinase
CKI: cyclin dependent kinase inhibitor
CLP: calmodulin like protein
EGF: epidermal growth factor
EGFR: epidermal growth factor receptor
ENU: N-nitroso-ethyl-urea
GSE: genetic suppressor element
HMEC: human mammary epithelial cells
HPV: human papilloma virus
LI: labeling index
p: passage
PaH: polycyclic aromatic hydrocarbon
PD: population doublings
PEM: polymorphic epithelial mucin
RB: retinoblastoma
TRF: terminal restriction fragment

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